

**REMARKS**

**The Amendments**

Claims 1-3 are amended to limit the scope of the claims in order to further prosecution. Applicants reserve the right to present the canceled parts of the claims in one or more continuation applications.

New Claims 19-21 are supported by, for example, page 5, lines 17-20 and page 29, Example 1.

No new matter is added in any of the amendments. The Examiner is requested to enter the amendment and reconsider the application.

**35 U.S.C. §112 Rejections**

In the Office Action, Claims 1-10 are rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for a method of reducing intraocular pressure comprising the administration of uridine 5'-diphosphate- $\alpha$ -D-glucose, does not allegedly provide enablement for a method of reducing intraocular pressure comprising the administration of any compound of Formula I.

The Examiner states that the only example in the specification is drawn to effects of uridine 5'-diphosphate- $\alpha$ -D-glucose on intraocular pressure; Applicants do not provide an adequate written description which provides guidance for the preparation or the use of compounds within the scope of Formula I.

The Examiner states that the data obtained from the uridine 5'-diphosphate- $\alpha$ -D-glucose examples are insufficient to provide adequate support for compounds of Formula I as broadly claimed or for their use in the instantly claimed method.

In order to expedite the allowance of this application, Applicants have amended the claims to substantially limit the claim scope. Applicants are submitting the following documents and arguments to show that the written description and enablement requirements are fulfilled for the claimed invention.

**A. Preparation of the compounds was known at the time of filing the application.**

In the application at page 23, first paragraph, it describes that:

The compounds of the present invention can be conveniently synthesized by those skilled in the art using well-known chemical procedures. Nucleoside 5'-pyrophosphate pyranose esters can be obtained from commercial sources or synthesized from the appropriate nucleoside and pyranose using a variety of phosphorylation and coupling reactions found in the chemical literature. Many different nucleosides, nucleotides, pyranose derivatives and inositol are commercially available and can be used as starting materials for these procedures.

Applicants have described the general methods of preparing the compounds at pages 23-26. Applicants are submitting herewith the following literature to illustrate that it was well known to a person skilled in the art how to prepare the compounds used in the instant claims, prior to the filing date of this application.

1. Sala et al., (*Carbohydrate Research* 1998, 306, 127-136)

The reference describes the synthesis of UDP-N-trifluoroacetylglucosamine using N-trifluoroacetylglucosamine-1-phosphate and UMP-morpholidate. The synthesis of this material is 5 steps long and the experimental section shows how to make and purify each of the intermediates, starting from commercially-available materials. Many references on the general synthesis of other related compounds like sugars, sugar phosphates, and nucleotide-sugars are provided. The synthesis itself uses an ester of this amido-sugar as an intermediate.

On page 129, paragraph 4 (bottom of page) the authors state "These conditions are known to produce .....  $\alpha$ -glycosyl phosphate diphenyl esters for a number of acetylated hexopyranoses" and references Sabesan and Neira (1992). This shows that there are many methods available to make derivatives of these materials.

2. Hanessian et al., (*J. Am. Chem. Soc.* 1998, 120, 13296-13300)

This reference describes the synthesis of UDP-galactose and UDP-glucose using non-enzymatic methods in this journal article. They also show how to synthesize diastereomers of hexose-1-phosphate derivatives using methoxypyridine ether intermediates. Authors give references to their previous work on the subject as a general method for synthesis of 2-OH and 2-N3 hexose derivatives. They also cite numerous references on the synthesis of sugar derivatives,

sugar phosphates, and nucleotide sugars.

Authors state on p.13297, paragraph 2 that “most nucleotide 5'-phosphates or diphosphates are easily accessible” and cite Hall, R.H.; Khorana, H.G. (*J. Am. Chem. Soc.* **1954**, *76*, 5056) to support their assertion.

Authors state on p. 13297, paragraph 3 that a “tetrabenzyl-glucopyranosyl bromide was condensed with UDP to give ...[first, tetrabenzyl ether diastereomers of UDP-glucose]... ,after deprotection, an anomeric mixture of ... UDP-glucose. The same technique was applicable to the synthesis of UDP-ara and UDP-fuc...”, indicating that this is a fairly general method of synthesizing compounds related to UDP-glucose.

Many intermediate hexose-1-phosphate derivatives were synthesized in this paper, and the authors present a general chemical synthesis method on page13299, 4<sup>th</sup> paragraph. General methods for purification and analysis are also mentioned in the 3<sup>rd</sup> paragraph on that page. More detailed purification and synthesis details are presented on pp13299-13300 for a variety of sugar phosphates and UDP-sugar derivatives, including ester derivatives, azido-sugars, amino derivatives of sugar phosphates.

3. Gegnas, et al. (*Bioorganic & Medicinal Chemistry Letters* **1998** *8*: 1643-1648)

This reference describes the synthesis of several ether derivatives of UDP-N-acetylglucosamine and (5-iodoUDP)- N-acetylglucosamine using a variety of chemical techniques. Many of the intermediates have acetals, amides, ethers, phosphate esters, and/or phosphates present, which indicate that these methods can be used to make a variety of nucleotide-diphospho-hexose derivatives related to UDP-glucose. Note also that in this article, a 5-halogen substituent can also be present on the uridine moiety, and the reaction sequences still work. In addition, these materials have an ether unit on the glucose 3-position. This points to the generality of the synthesis methodology used in the literature.

A number of references are cited for the OLD general synthetic processes that the authors used to make their NEW compounds. This also indicates that the older methods provide general experimental procedures that are useful for the synthesis of new, unknown compounds.

4. Simon, et al. (*J. Org. Chem.* **1990** *55*: 1834-1841)

This reference describes the synthesis and purification of UDP-glucose and GDP-mannose from commercially-available starting materials. The method reports using enzymes such as uridine-5'-diphosphoglucose pyrophosphorylase to catalyse the coupling of glucose phosphate and UTP, and reports on the sources of these enzymes on p. 1839, as well as on the isolation and purification of the materials. The synthesis of various phosphates and nucleotides is also reported, as are references on purification techniques to use.

5. Zervosen, et al., (*Tetrahedron*, 1996, 52, 2395-2404)

This reference reports the enzymatic synthesis of glucose derivatives of UDP, TDP, dUDP, ADP and CDP (p.2395, paragraph 2). These compounds could be made on a preparative scale. Full experimental details were provided for the enzymatic synthesis of 5 of these compounds, further indicating the generality of the method, especially considering that thymidine has a methyl group at the uridine 5-position, as well as a 2'-deoxy-group, and it still works.

6. Patent Abstracts of Japan (JP 07233187 A)

This reference describes the production of 5-fluorouridine 5'diphosphate-galactose.

7. Patent Abstracts of Japan (JP 60017000 A)

This reference describes the synthesis of 5-fluorouridine-5'diphosphate-glucose.

**B. Treatment Data of UDP-sugar derivative**

Applicants do not agree with the Examiner's statement that the treatment of ophthalmic conditions is unpredictable. Following Applicants' disclosure, a person skilled in the art is enabled to use the compounds claimed for reducing intraocular pressure in a subject.

Applicants have already demonstrated that UDP-glucose reduced intraocular pressure in rabbits (see Example 1). Applicants are submitting herewith a Declaration of Dr. Pintor under 37 CFR 1.132 to show that various UDP-sugar derivatives reduced intraocular pressure in rabbits.

In Dr. Pintor's Declaration, Figures 1-4 show the changes in IOP observed after the administration of UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, and (2'-deoxy-5-

methyl-UDP)-glucose, respectively. The results indicate that application of the above UDP-sugar derivatives reduced IOP by approximately 10%-30% within 1 to 4 hours following administration of each compound. In contrast, UDP-glucuronic acid (Figure 5), which is not included in Claim 1, failed to produce a significant decrease in IOP during the observation period of these studies in these rabbits.

Dr. Pintor's results show that nucleoside-diphopho-sugar derivatives that reduced IOP rabbit eyes include (a) UDP-glucose (Figure 1), (b) an isomer of UDP-glucose [i.e., UDP-galactose; (Figure 2)], (c) an acylamino analogue of UDP-glucose [i.e., UDP-(N-Acetyl)-glucosamine; (Figure 3)], and (d) a 5-alkyl-2'-deoxy-uridine analogue [i.e., TDP-glucose; (Figure 4)], which has two significant structural modifications, relative to UDP-glucose.

Therefore, the specification is enabled for the claimed invention.

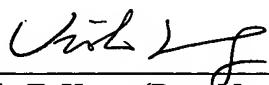
For the reasons states above, the rejection of Claims 11-18 under 35 USC 112, first paragraph should be withdrawn.

### CONCLUSION

Applicants believe that the application is in good and proper condition for allowance. Early notification of allowance is earnestly solicited. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is encouraged to call the undersigned at (650) 463-8181.

Respectfully submitted,

Date: May 19, 2004

  
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# UDP-*N*-trifluoroacetylglucosamine as an alternative substrate in *N*-acetylglucosaminyltransferase reactions

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## Abstract

The synthesis of UDP-*N*-trifluoroacetylglucosamine [uridine 5'-(2-trifluoroacetamido-2-deoxy- $\alpha$ -D-glucopyranosyl diphosphate, UDP-GlcNAc-F<sub>3</sub>]<sup>1</sup> is reported. The compound is found to serve as a substrate for the 'core-2' GlcNAc transferase (EC 2.4.1.102) that is involved in the biosynthesis of O-linked glycoproteins and for the GlcNAcT-V transferase (EC 2.4.1.155) that is a key biosynthetic enzyme controlling the branching pattern of cell surface complex Asn-linked oligosaccharides. The trisaccharide  $\beta$ -D-Galp-(1  $\rightarrow$  3)-[ $\beta$ -D-GlcNAc-F<sub>3</sub>(1  $\rightarrow$  6)]- $\alpha$ -D-GalpNAc-OR [R = (CH<sub>2</sub>)<sub>8</sub>CO<sub>2</sub>Me] was prepared from  $\beta$ -D-Galp-(1  $\rightarrow$  3)- $\alpha$ -D-GalpNAc-OR using the 'core-2' GlcNAc transferase. The tetrasaccharide  $\beta$ -D-GlcNAc-(1  $\rightarrow$  2)-[ $\beta$ -D-GlcNAc-F<sub>3</sub>-(1  $\rightarrow$  6)]- $\alpha$ -D-Manp-(1  $\rightarrow$  6)- $\beta$ -D-GlcNAc-OR [R = (CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>] was prepared from  $\beta$ -D-GlcNAc-(1  $\rightarrow$  2)- $\alpha$ -D-Manp-(1  $\rightarrow$  6)- $\beta$ -D-GlcNAc-OR [R = (CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>] using the GlcNAcT-V transferase. Removal of the trifluoroacetyl group was achieved under mild basic conditions to give the corresponding glucosamine containing tetrasaccharide. These examples demonstrate the feasibility of introducing masked forms of glucosamine residues into oligosaccharides using GlcNAc-specific transferases. The requirement for the trifluoroacetamido group as a specific recognition element was evident in the observation that neither UDP-glucosamine nor UDP-glucose served as a donor substrates for the 'core-2' GlcNAc transferase. © 1998 Elsevier Science Ltd. All rights reserved.

**Keywords:** *N*-Acetylglucosaminyltransferase; UDP-sugar analog; Oligosaccharide synthesis

## 1. Introduction

Cellular recognition in nature is often mediated via binding events involving complex carbohydrates on

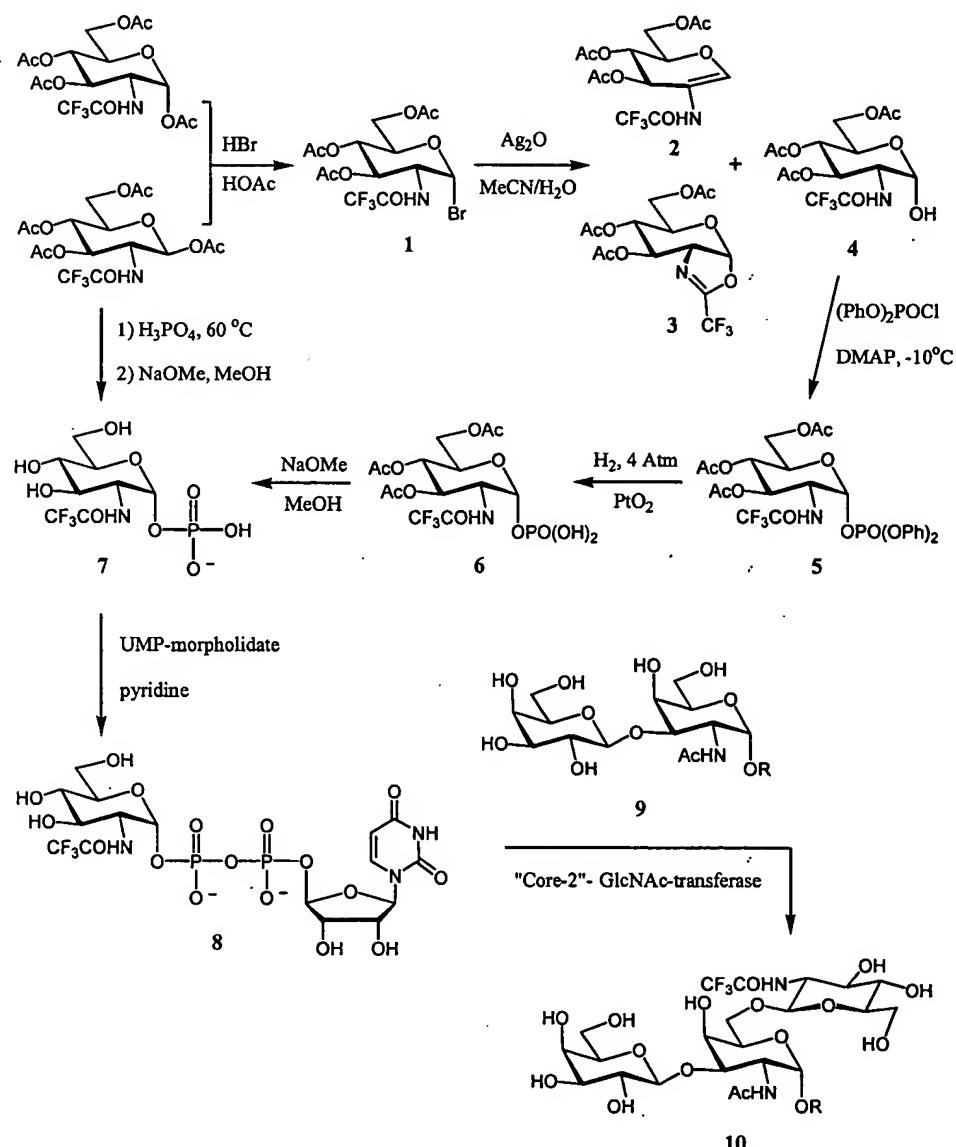
cell surfaces [1]. Compounds that interfere with these recognition processes are candidates as new drug targets in a variety of diseases. For this reason, strategies for the synthesis of oligosaccharides have received a great deal of attention in recent years [2–7]. One promising method involves the use of

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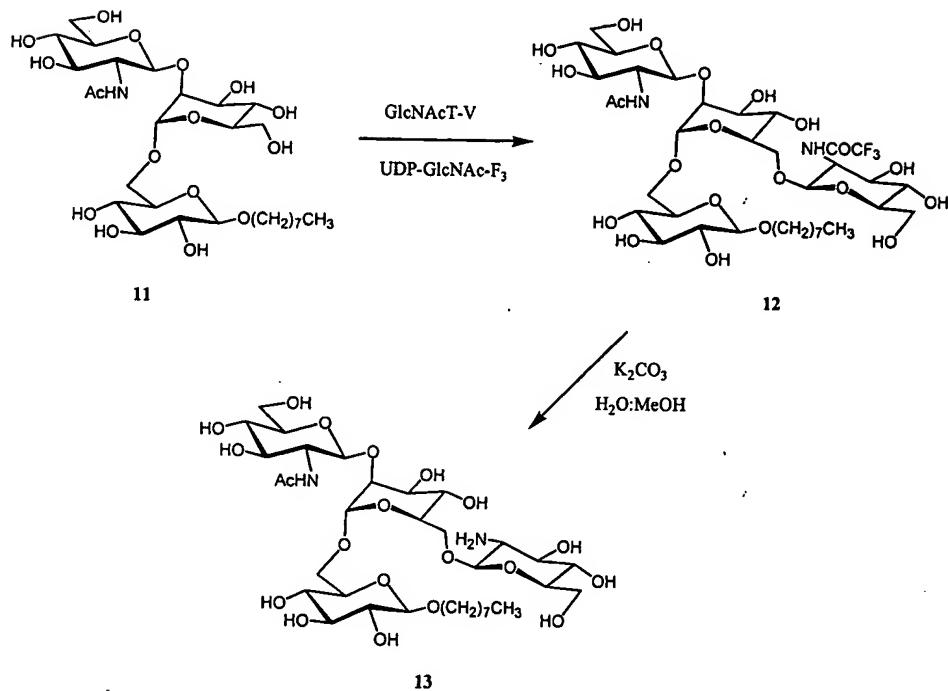
transferase enzymes that employ sugar-nucleotides as the carbohydrate donors [8–10]. The main advantage to this approach is that the enzymes are highly regiospecific and stereospecific with regard to directing the activated donor carbohydrate to the appropriate hydroxyl group of the acceptor, thereby eliminating the need for multiple protection–deprotection steps required in non-enzymatic syntheses. One of the drawbacks of this strategy, is that the specificity of these enzymes precludes the use of alternative donors that differ significantly from the natural donors. This makes the enzymatic synthesis of some unnatural oligosaccharides very difficult.

*N*-Acetylglucosamine (GlcNAc) residues are very

common components of natural glycoconjugates that are generated by a large class of transferases that utilize UDP-GlcNAc as a donor. The first of such enzymes to be discussed is the UDP-*D*-GlcNAc:  $\beta$ -*D*-Gal p-(1  $\rightarrow$  3)- $\alpha$ -*D*-GalpNAc (GlcNAc to GalNAc)  $\beta$ -(1  $\rightarrow$  6)-GlcNAc transferase that is also referred to as the ‘core-2’-GlcNAc transferase (GlcNAcT, EC 2.4.1.102) and is involved in the biosynthesis of O-linked glycoproteins in mammals [11,12]. This enzyme transfers a GlcNAc residue to the O-6 of GalNAc in the disaccharide  $\beta$ -*D*-Galp-(1  $\rightarrow$  3)- $\alpha$ -*D*-GalpNAc-OR (–OR can be either a hydroxyl residue of a serine or threonine in a protein or a simple aryl or alkyl aglycon). In this paper, we describe the



Scheme 1.



Scheme 2.

synthesis of UDP-*N*-trifluoroacetylglucosamine (UDP-GlcNAc-F<sub>3</sub>) and demonstrate that it can serve as an unnatural donor in the GlcNAcT reaction (Scheme 1). We also describe its recognition by the UDP-D-GlcNAc:  $\alpha$ -D-Manp-(1  $\rightarrow$  6)- $\beta$ -D-GlcNAc transferase V (GlcNAcT-V, EC 2.4.1.155). This enzyme transfers a GlcNAc residue to the O-6 of Man in the trisaccharide  $\beta$ -D-GlcNAc-(1  $\rightarrow$  2)- $\alpha$ -D-Manp-(1  $\rightarrow$  6)- $\beta$ -D-GlcNAc-OR (R = (CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>) (11) (Scheme 2) and controls the branching pattern of asparagine-linked oligosaccharides [13,14].

The ease of removal of the trifluoroacetyl group under relatively mild conditions [15–17] effectively permits the introduction of glucosamine residues into oligosaccharides that normally contain GlcNAc. This could lead to the preparation of a variety of unnatural compounds with potential bioactive properties as well as provide a nucleophilic functionality to which other molecules can be appended using reductive amination chemistry. The direct transfer of UDP-glucosamine was not expected to be feasible because the acetamido functionality of the natural substrate donor was being replaced by a charged ammonium group. Indeed, we have shown that the 'core-2'-GlcNAcT accepts neither UDP-glucose nor UDP-glucosamine as a donor substrate. This indicates the trifluoroacetamido group served as a necessary recognition element in the transferase reaction. In addition, we have

demonstrated the feasibility of this method by the enzymatic synthesis of the tetrasaccharide 12 followed by the removal of the trifluoroacetyl group to generate the glucosamine containing tetrasaccharide 13 (Scheme 2).

## 2. Results and discussion

The synthesis of UDP-*N*-trifluoroacetylglucosamine began with the preparation of the glycosyl bromide 1 from an  $\alpha$ / $\beta$  mixture of 1,3,4,5-tetra-*O*-acetyl-2-deoxy-2-trifluoroacetamido-D-glucose [18] (Scheme 1). Treatment of 1 with silver oxide in 10:1 acetonitrile:water gave 3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido- $\alpha$ -D-glucose 4 in 57% yield. The relatively low yield was due to the formation of the peracetylated glucal 2 and oxazoline 3 as by-products in yields of 7 and 12%, respectively. These products have been obtained previously upon treatment of 1 with MeOH under *Koenigs-Knorr* conditions [19]. Compound 4 was phosphorylated by treatment with diphenylchlorophosphate and 4-*N,N*-dimethylaminopyridine (DMAP) in CH<sub>2</sub>Cl<sub>2</sub> at  $-10^{\circ}\text{C}$ . These conditions are known to produce anomerically enriched  $\alpha$ -glycosyl phosphate diphenyl esters for a number of acetylated hexopyranoses [20]. Indeed the  $\alpha$ -diphenylphosphate 5 was obtained in 52% yield

Table 1  
Selected  $^1\text{H}$  NMR data<sup>a</sup> for compounds **9** [25] and **10**

Residue	Proton	<b>9</b>	<b>10</b>
GalNAc	H-1	4.88 (3.5)	4.84 (3.8)
	H-2	4.31 (3.5, 11.0)	4.29 (3.8, 11.1)
	H-3	4.02 (3.5, 11.0)	4.00 (3.1, 11.1)
	H-4	4.24 (2.9)	4.21 (3.4)
	NAc	2.02	2.00
$\beta$ Gal	H-1	4.48 (8.0)	4.46 (7.8)
	H-4	3.91 (3.0)	3.91 (3.4)
GlcNAc	H-1		4.63 (8.5)

<sup>a</sup>Numbers in parentheses give coupling constants in Hz.

and no  $\beta$ -anomer was isolated upon purification of the reaction product by conventional chromatography. A by-product ( $\sim 15\%$ ), whose  $^1\text{H}$  NMR and mass spectra were consistent with that expected for the corresponding  $\alpha$ -glycosyl chloride (3,4,6-tri- $O$ -acetyl-2-déoxy 2-trifluoroacetamido- $\alpha$ -D-glucopyranosyl chloride), was also obtained. The formation of glycosyl chlorides has been observed during the diphenylphosphorylation of sugars when *n*-butyllithium was used as the base [21]. The phenyl groups in the phosphate triester were removed by hydrogenation over platinum oxide catalyst [22] to give **6**. Low yields ( $\sim 30\%$ ) were observed, presumably due to acid catalyzed decomposition of the glycosyl phos-

phates. Cleavage of the  $O$ -acetyl groups in the presence of the base-sensitive trifluoroacetamido group was accomplished by careful treatment with NaOMe in MeOH to give **7** (68%).

In further work, a more efficient method for the preparation of compound **7** was developed. Treatment of the peracetylated  $\beta$ -N-trifluoroacetylglucosamine [18] with neat phosphoric acid at 60 °C (the MacDonald procedure [23]), followed by deacetylation with sodium methoxide in MeOH gave a 27% yield of **7**.

The GlcNAc-F<sub>3</sub>  $\alpha$ -phosphate **7** was coupled to UMP using standard Khorana conditions with UMP-morpholidate in dry pyridine [24]. The product UDP-GlcNAc-F<sub>3</sub> **8**, was isolated as its dilithium salt in

Table 2  
Selected  $^1\text{H}$  NMR data<sup>a</sup> for compounds **11** [26], **12** and **13**

Residue	Proton	<b>11</b>	<b>12</b>	<b>13</b>
$\beta$ -Glc	H-1	4.46 (8.1)	4.45 (8.1)	4.45 (8.1)
	H-2	3.26 (8.0, 9.2)	3.26 (8.5)	3.26 (8.7)
$\alpha$ -Man	H-1	4.90 (1.3)	4.82 (1.5)	4.90
	H-2	4.12 (3.4)	4.08 (3.5)	4.13 (3.5)
$\beta$ -GlcNAc	H-1	4.57 (8.4)	4.56 (8.4)	4.56 (8.4)
	NAc	2.06	2.04	2.07
$\beta$ -GlcNAc-F <sub>3</sub>	H-1		4.64 (8.4)	
$\beta$ -GlcNH <sub>2</sub>	H-1			4.39 (8.1)
	H-2			2.68 (9.0)

<sup>a</sup>Numbers in parentheses give coupling constants in Hz.

44% yield following purification by anion exchange and size exclusion chromatographies.

'Core-2'-GlcNAc-transferase was obtained from mouse kidney acetone powder by extraction and digestion with trypsin followed by a single step affinity chromatography on UDP-hexanolamine Sepharose (see Section 3). Incubation of  $\beta$ -D-Galp-(1  $\rightarrow$  3)- $\alpha$ -D-GalpNAc-OR **9** [25] with a portion of this enzyme extract, in the presence of the donor UDP-GlcNAc-F<sub>3</sub>, resulted in the formation of the trisaccharide product **10** in a 62% isolated yield.

The structure of **10** was assigned on the basis of the <sup>1</sup>H NMR data showed in Table 1. The new  $\beta$ -glycosidic linkage was established by the H-1 doublet ( $J_{1,2}$  8.5 Hz) at  $\delta$  4.63 ppm. This was in agreement with a previous assignment of the *N*-acetylglucosamine analog of this trisaccharide [11]. High resolution electrospray ionization mass spectrometry (HRESIMS) confirmed the composition and size of **10** ( $[M + Na]^+ = 833.3173$ ). The presence of the *N*-trifluoroacetyl group was confirmed by the singlet at  $\delta$  –76.1 ppm in the <sup>19</sup>F NMR.

The trifluoro donor was also incubated with a portion of cloned GlcNAcT-V transferase and acceptor **11**. The resultant tetrasaccharide **12** was obtained in a 56% isolated yield. From the <sup>1</sup>H NMR data presented in Table 2, the new  $\beta$ -glycosidic linkage of **12** was confirmed by the H-1 doublet ( $J_{1,2}$  8.4 Hz) at  $\delta$  4.64 ppm. The composition of **12** was confirmed by the  $[M + Na]^+$  peak at 937.3622 in the HRESIMS spectrum. The presence of the *N*-trifluoroacetyl group was confirmed by the singlet at  $\delta$  –75.5 in the <sup>19</sup>F NMR.

The preparation of **10** and **12** clearly demonstrates the feasibility of using enzymes to introduce a protected form of glucosamine into an oligosaccharide. In order to show that the trifluoroacetamido group was a specific recognition component in the enzymatic reaction, the alternative substrates UDP-glucosamine (bearing a small positively charged group at C-2) and UDP-glucose (bearing a small neutral group at C-2) were tested as substrates. In either case, no detectable transfer was observed after eight days. The presence of an acetamido moiety at C-2 was therefore required for the transfer to occur; as one might expect for these highly specific transferases.

Deprotection of tetrasaccharide **12** was performed using a previously published procedure [17] which employed potassium carbonate in aqueous MeOH. These mild conditions allowed the isolation of the desired tetrasaccharide **13** after purification by reversed-phase chromatography.

### 3. Experimental

**General methods.**—Melting points were recorded on a Reichert melting point apparatus and were uncorrected. Optical rotations were measured with a Perkin-Elmer 241 MC polarimeter. TLC was performed on Silica Gel 60F<sub>254</sub> (E. Merck, Darmstadt) with detection by charring after spraying with 5% H<sub>2</sub>SO<sub>4</sub> or by spraying with a solution containing H<sub>2</sub>SO<sub>4</sub> (31 mL) ammonium molybdate (21 g), and cerium sulfate (1 g) in water (500 mL) and then heating (110 °C for 5 min). Flash column chromatography was performed using Silica Gel 60 (230–400 mesh). All reagents were commercially available unless otherwise stated. The disaccharide  $\beta$ -D-Galp-(1  $\rightarrow$  3)- $\alpha$ -D-GalpNAc-OR [R = (CH<sub>2</sub>)<sub>8</sub>CO<sub>2</sub>Me] (**9**) and the trisaccharide  $\beta$ -D-GlcNAc-(1  $\rightarrow$  2)- $\alpha$ -D-Manp-(1  $\rightarrow$  6)- $\beta$ -D-GlcP-OR [R = (CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>] (**11**) were respectively synthesized as previously described [25,27]. UDP-GlcNAc was obtained from Sigma (St. Louis, MO). UDP-[6-<sup>3</sup>H(N)]GlcNAc (specific activity, 60 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). Liquid scintillation counting was performed with a Beckman LS-1801 instrument using Ecolite (+) cocktail from ICN Radiochemicals (Costa Mesa, CA). Iatrobeads refers to a beaded Silica Gel (No. 6RS-8060) manufactured by Iatron Laboratories (Tokyo). Ion exchange and gel chromatographic supports were from BioRad Laboratories (Missisauga, ON). Pyridine, acetonitrile, CH<sub>2</sub>Cl<sub>2</sub>, and Et<sub>3</sub>N were distilled under N<sub>2</sub> from calcium hydride. Methanol was distilled under N<sub>2</sub> from magnesium methoxide. All reactions performed in organic solvents were carried out under argon. <sup>1</sup>H NMR spectra were recorded at 400 MHz (Bruker WH-400) or at 600 MHz (Varian spectrometer) in solutions in deuterium oxide with acetone as the external standard ( $\delta$  2.23 ppm) and at 30 °C (for compounds **9** and **13**). <sup>13</sup>C NMR spectra were recorded at 75.43 MHz (Varian XL-300), <sup>31</sup>P NMR spectra were recorded at 81.0 MHz and <sup>19</sup>F NMR at 188.0 MHz with proton decoupling (Bruker AC-200). The <sup>31</sup>P chemical shifts are expressed relative to external phosphoric acid (0.00 ppm). The <sup>19</sup>F chemical shifts are expressed relative to CFCl<sub>3</sub> for solutions in CDCl<sub>3</sub> (0.00 ppm) or AcOH for solutions in deuterium oxide (–76.53 ppm). Signals upfield of CFCl<sub>3</sub> were assigned negative values. Desorption chemical ionization (DCI) mass spectra were recorded on a Delsi Nermag R10-10C mass spectrometer using ammonia as the chemical ionization gas. High resolution liquid secondary ion (HRLSI) mass spectra were

recorded on a Kratos Concept II HQ mass spectrometer. High resolution electrospray ionization (HRESI) mass spectrometry of **10** was obtained using a Micro-mass ZabSpec Hybrid Sector-TOF spectrometer using a 1% solution of ArOH in water:MeOH (1:1) as liquid carrier. Millex-GV (0.22 mm) filters units were from Millipore, and C<sub>18</sub> Sep-Pak sample-preparation cartridges were from Waters Associates. Microanalysis were carried out by Mr. Peter Borda in the microanalytical laboratory at UBC.

**3,4,6-Tri-O-acetyl-2-deoxy-2-trifluoroacetamido- $\alpha$ -D-glucose (4).**—3,4,6-Tri-O-acetyl-2-deoxy-2-trifluoroacetamido- $\alpha$ -D-glucopyranosyl bromide **1** (1.08 g, 2.3 mmol) was dissolved in 10 mL of dry acetonitrile containing silver oxide (0.50 g), water (1 mL) was added, and the mixture was stirred for 2 h in the dark. The resulting suspension was filtered, concentrated, and separated on a silica gel column using 1:3 EtOAc–hexanes. Fractions of higher mobility were segregated for further purification. Later fractions were pooled and evaporated to give **4** as a white solid. (0.53 g, 57%): mp 174 °C, lit. 173–174 °C [18];  $[\alpha]_D^{22} + 20.0^\circ$  (c 0.5, CHCl<sub>3</sub>), lit. + 25.0° (c 1, CHCl<sub>3</sub>) [18];  $R_f$  0.16 (1:1 EtOAc–hexanes); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  6.64 (br d, 1 H,  $J_{2,NH}$  9.1 Hz, NH), 5.34 (dd, 1 H,  $J_{2,3}$  9.9,  $J_{3,4}$  9.6 Hz, H-3), 5.33 (d, 1 H,  $J_{1,2}$  3.3 Hz, H-1), 5.13 (dd, 1 H,  $J_{4,5}$  9.6 Hz, H-4), 4.29 (ddd, H-2) 4.25–4.08 (m, 3 H, H-5, H-6, H-6') 2.08, 2.03, 2.00 (3s, each 3 H, 3Ac); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  171.40, 171.05, 169.48 (COCH<sub>3</sub>, 3Ac), 157.30, (q, COCF<sub>3</sub>,  $J_{C,F}$  37.7 Hz), 115.53 (q, CF<sub>3</sub>,  $J_{C,F}$  287.7 Hz), 90.87 (C-1), 70.46 (C-5), 67.82 (C-3), 67.71 (C-4), 61.89 (C-6), 52.76 (C-2), 20.77, 20.60, 20.48 (CH<sub>3</sub>, 3Ac); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$  –76.6; DCIMS *m/z* 419 ([M + NH<sub>4</sub>]<sup>+</sup>, 100%). Anal. Calcd. for C<sub>14</sub>H<sub>18</sub>O<sub>9</sub>NF<sub>3</sub>: C, 41.90; H, 4.52; N, 3.49. Found: C, 42.15; H, 4.32; N, 3.29.

**3,4,6-Tri-O-acetyl-5-anhydro-2-deoxy-2-trifluoroacetamido-D-arabino-hex-1-enitol (2).**—Obtained in 7% yield as a by-product of the chromatographic purification of **4**. Further purification by flash chromatography using 9:1 benzene–ether was required in order to separate this compound from the oxazoline **3**. Clear oil,  $[\alpha]_D^{22} - 46.5^\circ$  (c 1, CHCl<sub>3</sub>), lit. –45° (c 1, CHCl<sub>3</sub>) [18];  $R_f$  0.25 (7:3 benzene–ether); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  8.19 (br s, 1 H, NH), 7.69 (s, 1 H, H-1), 5.22 (dd, 1 H,  $J_{3,4} = J_{4,5}$  3.6 Hz, H-4), 5.15 (dd, 1 H,  $J_{3,5}$  1.1 Hz, H-3), 4.45–4.40 (ddd, 1 H,  $J_{5,6}$  7.9,  $J_{5,6'}$  4.1 Hz, H-5), 4.35 (dd, 1 H,  $J_{gem}$  11.9 Hz, H-6), 4.22 (dd, 1 H, H-6') 2.12, 2.10, 2.08 (3s, each 3 H, 3Ac); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  172.66, 170.48, 169.54 (COCH<sub>3</sub>, 3Ac), 154.73 (q,

$J_{C,F}$  37.5 Hz, COCF<sub>3</sub>), 115.53 (q,  $J_{C,F}$  286.4 Hz, CF<sub>3</sub>), 141.07 (C-1) 128.34 (C-2) 73.19, 66.40, 66.32, 60.92 (C-3, C-4, C-5, C-6) 20.82, 20.79, 20.75 (CH<sub>3</sub>, 3Ac); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$  –76.4; DCIMS *m/z* 401 ([M + NH<sub>4</sub>]<sup>+</sup>, 100%). Anal. Calcd. for C<sub>14</sub>H<sub>16</sub>O<sub>8</sub>NF<sub>3</sub>: C, 43.87; H, 4.21; N, 3.65. Found: C, 43.58; H, 4.28; N, 3.90.

**2-Trifluoromethyl-(3,4,6-tri-O-acetyl-1,2-dideoxy- $\alpha$ -D-glucopyranosyl)[2,1-D]- $\Delta^2$ -oxazo line (3).**—Obtained as a clear oil as described above;  $[\alpha]_D^{22} + 3.6^\circ$  (c 2.2, CHCl<sub>3</sub>), lit. + 5.5° (c 2, CHCl<sub>3</sub>) [18];  $R_f$  0.32 (7:3 benzene–ether); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  6.27 (d, 1 H,  $J_{1,2}$  7.6 Hz, H-1), 5.31 (dd, 1 H,  $J_{2,3} = J_{3,4}$  2.4 Hz, H-3), 4.93 (ddd, 1 H,  $J_{2,4}$  1.3,  $J_{4,5}$  8.2 Hz, H-4), 4.36 (m, 1 H, H-2) 4.28–4.15 (m, 2 H, H-6, H-6'), 3.63 (m, 1 H, H-5), 2.10, 2.08, 2.06 (3s, each 3, 3Ac); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  170.60, 169.33, 169.02 (COCH<sub>3</sub>, 3Ac), 156.22 (q,  $J_{C,F}$  41.0 Hz, CNCF<sub>3</sub>), 115.90 (q,  $J_{C,F}$  273.0 Hz, CF<sub>3</sub>), 102.22 (C-1), 68.77, 68.60, 67.51, 63.97, 63.20 (C-2, C-3, C-4, C-5, C-6) 20.77, 20.63, 20.62 (CH<sub>3</sub>, 3Ac); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$  –71.3; DCIMS *m/z* 401 ([M + NH<sub>4</sub>]<sup>+</sup>, 100%). Anal. Calcd. for C<sub>14</sub>H<sub>16</sub>O<sub>8</sub>NF<sub>3</sub>: C, 43.87; H, 4.21; N, 3.65. Found: C, 44.11; H, 4.19; N, 3.57.

**Diphenyl (3,4,6-tri-O-acetyl-2-deoxy-2-trifluoroacetamido- $\alpha$ -D-glucopyranosyl) phosphate (5).**—A mixture of **4** (1.24 g, 3.1 mmol) and DMAP (0.87 g, 7.1 mmol) in 25 mL of dry CH<sub>2</sub>Cl<sub>2</sub> was stirred at room temperature for 10 min and then cooled to –10 °C. Diphenylchlorophosphate (1.4 mL, 7.1 mmol) was added dropwise and the solution was stirred at this temperature for 2 h. The mixture was then diluted with CH<sub>2</sub>Cl<sub>2</sub> (30 mL) and washed with ice-cold water, ice-cold 0.5 M HCl and saturated NaHCO<sub>3</sub>. After drying the organic phase over MgSO<sub>4</sub>, it was concentrated to a small residue which was purified by column chromatography using 2:3 EtOAc–hexanes to afford the title compound as white crystals (1.02 g, 52%): mp 65 °C;  $[\alpha]_D^{20} + 60.0^\circ$  (c 1, CHCl<sub>3</sub>);  $R_f$  0.27 (2:3 EtOAc–hexanes); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.40–7.08 (m, 10 H, 2Ph), 6.88 (br d, 1 H,  $J_{2,NH}$  8.7 Hz, NH), 6.01 (dd, 1 H,  $J_{H,P}$  6.0 Hz,  $J_{1,2}$  3.2 Hz, H-1), 5.32 (dd, 1 H,  $J_{3,4}$  9.9,  $J_{2,3}$  11.3 Hz, H-3), 5.20 (dd, 1 H,  $J_{4,5}$  9.7 Hz, H-4), 4.41 (ddd, 1 H, H-2), 4.20–3.88 (m, 3 H, H-5, H-6, H-6'), 2.03, 2.01, 1.99 (3s, each 3 H, 3Ac); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  170.92, 170.54, 169.21 (COCH<sub>3</sub>, 3Ac), 157.77 (q,  $J_{C,F}$  38.4 Hz, COCF<sub>3</sub>), 129.96, 129.89, 125.85, 125.80, 119.91, 119.85, 119.82, 119.76 (2Ph), 115.45 (q,  $J_{C,F}$  287.6 Hz, CF<sub>3</sub>), 96.12 (d,  $J_{C,P}$  7 Hz, C-1), 70.03 (C-5), 69.39 (C-3), 67.20 (C-4), 60.91 (C-6), 52.48 (d,  $J_{C,P}$  8.4 Hz, C-2), 20.53, 20.45, 20.24

(CH<sub>3</sub>, 3Ac); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$  –76.1; <sup>31</sup>P NMR (CDCl<sub>3</sub>):  $\delta$  –14.39; DCIMS *m/z* 651 ([M + NH<sub>4</sub>]<sup>+</sup>, 100%). Anal. Calcd. for C<sub>26</sub>H<sub>27</sub>O<sub>12</sub>NPF<sub>3</sub>: C, 49.30; H, 4.30; N, 2.21. Found: C, 49.08; H, 4.41; N, 2.22.

*Monotriethylammonium salt of 3,4,6-tri-O-acetyl-2-deoxy-2-trifluoroacetamido- $\alpha$ -D-glucopyranosyl phosphate (6).*—A solution of 5 (250 mg, 0.4 mmol) in 1:1 EtOAc–MeOH (10 mL) was hydrogenated (4 atm) in the presence of platinum oxide catalyst (25 mg). After 4 h, the catalyst was removed by filtration and Et<sub>3</sub>N (2 mL) was added to the filtrate. Evaporation of the solvent afforded a syrup that was purified by preparative thin layer chromatography using 10:10:1 CHCl<sub>3</sub>–MeOH–water to give the free acid form of 6 as a white hygroscopic solid (60 mg, 32%), this was converted to the monotriethylammonium salt by passing through a small column (3 cm) of Dowex 50W-X8 (triethylammonium form):  $R_f$  0.6 (10:10:1 CHCl<sub>3</sub>–MeOH–water); <sup>1</sup>H NMR (deuterium oxide):  $\delta$  5.53 (dd, 1 H,  $J_{1,P}$  7.1 Hz,  $J_{1,2}$  3.1 Hz, H-1), 5.42 (dd, 1 H,  $J_{2,3}$  10.4,  $J_{3,4}$  9.8 Hz, H-3), 5.13 (dd, 1 H,  $J_{4,5}$  9.8 Hz, H-4), 4.50–4.14 (m, 4 H, H-2, H-5, H-6, H-6') 2.14, 2.10, 2.02 (3s, each 3 H, 3Ac); <sup>13</sup>C NMR (deuterium oxide):  $\delta$  174.07, 173.43, 173.14 (COCH<sub>3</sub>, 3Ac), 159.64 (q,  $J_{C,F}$  38.4 Hz, COCF<sub>3</sub>), 115.86 (q,  $J_{C,F}$  284.5 Hz, CF<sub>3</sub>), 92.79 (d,  $J_{C,P}$  5.5 Hz, C-1), 71.17 (C-5), 68.63 (C-3), 68.31 (C-4), 62.05 (C-6), 52.57 (d,  $J_{C,P}$  8.4 Hz, C-2), 20.41, 20.37, 20.20 (CH<sub>3</sub>, 3Ac); <sup>19</sup>F NMR (deuterium oxide):  $\delta$  –75.4; <sup>31</sup>P NMR (deuterium oxide):  $\delta$  –1.07; HRLSIMS *m/z*: 480.0522 (calcd. for C<sub>14</sub>H<sub>19</sub>O<sub>12</sub>NPF<sub>3</sub>, 480.0519).

*Monopyridinium salt of 2-deoxy-2-trifluoroacetamido- $\alpha$ -D-glucopyranosyl phosphate (7).*—Compound 6 (180 mg, 0.4 mmol) was dissolved in 3 mL of dry MeOH. The solution was cooled to 0 °C and 0.50 mL of NaOMe in MeOH (1 M) was added. After stirring for 20 min, the reaction was quenched using Dowex 50W-X8 resin (pyridinium form, previously washed with MeOH). The resin was filtered off and the methanolic solution was evaporated. The residue was dissolved in a small amount of water, passed through a small column (3 cm) of Dowex 50W-X8 (pyridinium form) resin and lyophilized to give 7 as the monopyridinium salt (120 mg, 68%): <sup>1</sup>H NMR (deuterium oxide):  $\delta$  5.48 (dd, 1 H,  $J_{1,P}$  7.2,  $J_{1,2}$  3.2 Hz, H-1), 4.04 (dd, 1 H,  $J_{2,3}$  10.6 Hz, H-2), 3.93–3.72 (m, 4 H, H-3, H-5, H-6, H-6'), 3.53 (dd, 1 H,  $J_{3,4} = J_{4,5}$  9.6 Hz, H-4); <sup>13</sup>C NMR (deuterium oxide):  $\delta$  159.61 (q,  $J_{C,F}$  37.6 Hz, COCF<sub>3</sub>), 115.96 (q,  $J_{C,F}$  284.7 Hz, CF<sub>3</sub>), 93.12 (d,  $J_{C,P}$  5.8 Hz, C-1), 73.04 (C-5), 70.20 (C-3), 69.80 (C-4),

60.50 (C-6), 54.65 (d,  $J_{C,P}$  8.0 Hz, C-2); <sup>19</sup>F NMR (deuterium oxide):  $\delta$  –75.7; <sup>31</sup>P NMR (deuterium oxide):  $\delta$  –1.33; HRLSIMS *m/z* 354.0189 (calcd. for C<sub>8</sub>H<sub>12</sub>O<sub>9</sub>NPF<sub>3</sub>: 354.0202).

*Preparation of 7 by the MacDonald procedure.*—Crystalline phosphoric acid (500 mg, 5.1 mmol) was dried in vacuo over phosphorus pentoxide for 12 h. Solid 1,3,4,6-tetra-O-acetyl-2-deoxy-2-trifluoroacetamido- $\beta$ -D-glucopyranose (280 mg, 0.63 mmol) was added and the mixture was heated at 60 °C in vacuo. The formation of a melt and the evolution of CH<sub>3</sub>COOH vapors was observed. After 2 h, heating was ceased and the resulting dark black mixture was dissolved in anhydrous THF (5 mL). The solution was cooled to 0 °C and concentrated ammonium hydroxide (0.5 mL) was added. The precipitate of ammonium phosphate was filtered off and washed with THF (20 mL). The combined filtrate and washings were evaporated to give a syrupy residue that was applied to two plates of silica gel (2 mm thickness) and eluted with 10:10:1 CHCl<sub>3</sub>–MeOH–water. Isolation of the corresponding zone gave crude 6 as a light brown solid. This material was O-deacetylated and purified as described above to give 7 (73 mg, 27%).

*Dilithium uridine-5'-(2-deoxy-2-trifluoroacetamido- $\alpha$ -D-glucopyranosyl diphosphate) (8).*—A solution of 4-morpholine N,N'-dicyclohexylcarboxamidinium uridine 5'-phosphomorpholidate (210 mg, 0.31 mmol) in anhydrous pyridine (5–7 mL) was concentrated to dryness in vacuo. The process of dissolution in fresh dry pyridine and concentration was repeated twice, dry air being admitted into the flask after each concentration. Separately, an aqueous solution of the monopyridinium salt of sugar phosphate 7 (100 mg, 0.22 mmol) was treated similarly. A solution of the residue in pyridine was added to the dry phosphomorpholidate described above. This solution was concentrated in vacuo and redissolved in ~5 mL of dry pyridine. The resulting solution was sealed, kept at room temperature for 5 days and then concentrated. The residual syrup was redissolved in 10 mL of water, applied onto a column of Dowex 1X4-200 (chloride form, strongly basic), and eluted with a gradient of lithium chloride (0.1–1 M) at pH 3.5. The sugar nucleotide containing fractions were pooled, concentrated to a small volume, and desalted by passing through a column of Bio-Gel P-2 column (2.5 × 45 cm) eluted with water. The appropriate fractions were pooled and lyophilized to give 67 mg (44%) of 8 as its dilithium salt (dihydrate): <sup>1</sup>H NMR (deuterium oxide):  $\delta$  7.92 (d, 1 H,  $J_{5,6}$  8.1 Hz, H-6),

5.93 (d, 1 H,  $J_{1',2'} 4.9$  Hz, H-1'), 5.91 (d, 1 H, H-5), 5.57 (dd, 1 H,  $J_{1'',p} 7.1$ ,  $J_{1'',2''} 3.3$  Hz, H-1''), 4.34–4.28 (m, 2 H, H-2', H-3'), 4.25–4.12 (m, 3 H, H-4'', H-5'a, H-5'b), 3.95–3.72 (m, 4 H, H-2'', H-5'', H-6''a, H-6''b, H-3''), 3.54 (dd, 1 H,  $J_{3'',4''} = J_{4'',5''} 9.6$  Hz, H-4'');  $^{13}\text{C}$  NMR (deuterium oxide):  $\delta$  160.4 (q,  $J_{\text{C},\text{F}}$  37.7 Hz,  $\text{COCF}_3$ ), 167.2 (C-4), 152.7 (C-2), 142.5 (C-6), 116.6 (q,  $J_{\text{C},\text{F}}$  284.4 Hz,  $\text{CF}_3$ ), 103.5 (C-5), 94.6 (d,  $J_{\text{C},\text{P}}$  5.9 Hz, C-1''), 89.3 (C-1'), 84.1 (d,  $J_{\text{C},\text{P}}$  9.2 Hz, C-4'), 74.7 (C-3'), 73.9 (C-5''), 71.2 (C-2''), 70.5, 70.3 (C-3'', C-4''), 65.8 (d,  $J_{\text{C},\text{P}}$  5.5 Hz, C-5''), 61.1 (C-6''), 55.3 (d,  $J_{\text{C},\text{P}}$  8.8 Hz, C-2'');  $^{19}\text{F}$  NMR (deuterium oxide):  $\delta$  –74.9;  $^{31}\text{P}$  NMR (deuterium oxide):  $\delta$  –11.01 (d,  $J$  20.3 Hz,  $\text{P}\beta$ ), –12.90 (d,  $J$  20.3 Hz,  $\text{P}\alpha$ ); HRLSIMS  $m/z$  674.07928 (calcd. for  $\text{C}_{17}\text{H}_{23}\text{O}_{17}\text{N}_3\text{P}_2\text{F}_3\text{Li}_2$ , 674.0775). Anal. Calcd. for  $\text{C}_{17}\text{H}_{22}\text{N}_3\text{O}_{17}\text{P}_2\text{F}_3\text{Li}_2 \cdot 2\text{H}_2\text{O}$ : C, 28.79; H, 3.70; N, 5.92. Found: C, 28.68; H, 3.79; N, 5.71.

*Preparation of the glycosyltransferase 'core-2'-GlcNAcT (UDP-D-GlcNAc:β-D-Gal-(1 → 3)-α-D-GalpNAc - β - (1 → 6) - GlcNAc transferase (EC 2.4.1.102)).*—Mouse kidney acetone powder (1 g, Sigma) was suspended in 25 mL NaOAc buffer (A: 0.1 M NaOAc, pH 6.0; 0.2 M NaCl, 0.01 M EDTA) containing 12 mg trypsin and stirred vigorously overnight at 4 °C. The slurry was centrifuged at 6000 × g for 20 min at 4 °C. Soybean trypsin inhibitor (5 mg) was added to the supernatant which was stored at 4 °C. The pellet was resuspended in 25 mL of buffer A containing 12 mg of trypsin and stirred vigorously overnight at 4 °C. This preparation was centrifuged at 6000 × g for 20 min at 4 °C. The addition of 5 mg of soybean trypsin inhibitor to the combined supernatants was followed by the overnight dialysis versus 1 L of HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid) buffer (B: 0.05 M HEPES, pH 6.5; 0.005 M manganese(II) chloride, 0.1% 2-mercaptoethanol). Dialysis tubing with a molecular weight cutoff of 12 000–14 000 Da was used. The dialysate was centrifuged at 100 000 × g for 30 min at 4 °C and the supernatant was then assayed for GlcNAcT activity (~170 mU). This crude extract was then loaded, at a flow rate of 0.15 mL/min, onto a 20 mL UDP-hexanolamine Sepharose 4B gel (3.4  $\mu\text{mol}/\text{mL}$ ) column which had previously been equilibrated with buffer B. The column was then washed with buffer B (40 mL) and subsequently eluted with 30 mL of 0.5 M NaCl buffer (C: 0.05 HEPES, pH 6.5; 0.5 M NaCl, 0.1% 2-mercaptoethanol). Fractions (1 mL) were collected in plastic tubes and assayed for 'core-2' activity. The fractions containing the highest transferase activity

(~119 mU/6 mL) were used for preparative glycosylations.

*Radiochemical assay for GlcNAcT.*—GlcNAcT activity was measured using a modified version of a previously published procedure [11]. Each assay tube contained: 1.6  $\mu\text{L}$  of 500 mM GlcNAc solution, 1.0  $\mu\text{L}$  of 10 mM UDP-GlcNAc, 1.5  $\mu\text{L}$  of a 3.6 mM solution of acceptor 1, 0.5  $\mu\text{L}$  of UDP-6-[ $^3\text{H}$ ]GlcNAc (~40 000 dpm), and 7.5  $\mu\text{L}$  of HEPES buffer (0.2 mM HEPES, pH 6.5; 2% Triton X-100). To this total of 12.1  $\mu\text{L}$  was added 7.9  $\mu\text{L}$  of crude or diluted enzyme sample. The mixture was incubated for 90 min at 37 °C, diluted with approximately 3 mL of water and loaded onto a  $\text{C}_{18}$  Sep-Pak cartridge which had been pre-equilibrated with water. The cartridge was washed with 50 mL of water and the product eluted into a scintillation vial with 4 mL of MeOH. The radioactivity of the eluants were quantitated by liquid scintillation counting using 10 mL of scintillation cocktail.

*Enzymatic conversion of β-Gal-(1 → 3)-α-GalNAc-OR (9) into β-Gal-(1 → 3)-[β-GlcNAc-F<sub>3</sub> (1 → 6)]-α-GalpNAc - OR (10).*—UDP-N-trifluoroacetylglucosamine (0.46 mg, 0.64  $\mu\text{mol}$ ), 9 (0.8 mg, 1.45  $\mu\text{mol}$ ), bovine serum albumin (25  $\mu\text{L}$  of a 100 mg/mL aqueous solution) and alkaline phosphatase from calf intestine (1 U), were added to a 1.5 mL microcentrifuge tube. This was followed by the addition of 490  $\mu\text{L}$  of GlcNAcT (14 mU) and 474  $\mu\text{L}$  of 50 mM HEPES buffer, pH 6.5 containing 0.1% 2-mercaptoethanol. The reaction mixture was incubated at room temperature. Additional donor (0.38 mg, 0.53  $\mu\text{mol}$ ) was added after 48 h. After 96 h donor (0.3 mg, 0.42  $\mu\text{mol}$ ) and alkaline phosphatase (0.5 U) were added to the reaction. After 8 days, the reaction was worked up by loading it onto two pre-washed  $\text{C}_{18}$  Sep-Pak cartridges. The cartridges were washed with 30 mL of water and the crude product eluted with 30 mL of MeOH. The MeOH eluant was evaporated to dryness and the residue chromatographed on an Iatrobead-column with 30:20:1  $\text{CH}_2\text{Cl}_2$ –MeOH–water. The product fractions were pooled, concentrated, dissolved in water and filtered through a 0.22  $\mu\text{m}$  filter before lyophilization. Trisaccharide 10 was obtained in 62% (0.73 mg) isolated yield. Partial  $^1\text{H}$  NMR assignments are reported in Table 1. HRESIMS  $m/z$  833.3173 (calcd. for  $\text{C}_{32}\text{H}_{53}\text{N}_2\text{O}_{18}\text{F}_3\text{Na}$ : 833.3143).

UDP-glucose and UDP-glucosamine were used as donors in the above reaction but no transfer was observed after 8 days.

*Glucosyltransferase GlcNAcT - V [UDP - D -*

*GlcNAc- $\alpha$ -D-Manp-(1 $\rightarrow$ 6)- $\beta$ -D-GlcNAc-V transferase (GlcNAcT-V, EC 2.4.1.155)].*—The 97 kDa GlcNAcT-V transferase was cloned from rat kidney [27] and was a generous gift from Dr. M. Pierce, Department of Biochemistry, University of Georgia.

*Radiochemical assay for GlcNAcT-V.*—Enzyme activity was monitored using a modified version of a previously published procedure [28]. Each assay tube contained: 8.0  $\mu$ L of 1.38 mM UDP-GlcNAc, 1.0  $\mu$ L of a 0.8 mM solution of acceptor **11**, 1.0  $\mu$ L of UDP-6-[ $^3$ H]GlcNAc ( $\sim 10^5$  dpm), and 5.0  $\mu$ L of sodium cacodylate buffer (100 mM sodium cacodylate, pH 6.5; 20 mM EDTA; 40% glycerol; 2 mg/mL bovine serum albumin). The work-up of the incubated samples was performed in the same manner as given previously for the radiochemical assay for GlcNAcT.

*Enzymatic conversion of  $\beta$ -D-GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -D-Manp-(1 $\rightarrow$ 6)- $\beta$ -D-Glc-OR (R = (CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>) (**11**) into  $\beta$ -D-GlcNAc-(1 $\rightarrow$ 2)-[ $\beta$ -D-GlcNAc-F<sub>3</sub>-(1 $\rightarrow$ 6)]- $\alpha$ -D-Manp-(1 $\rightarrow$ 6)- $\beta$ -D-Glc-OR (R = (CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>) (**12**).*—UDP-N-trifluoroacetylglucosamine (2.4 mg, 3.34  $\mu$ mol), **11** (4.2 mg, 6.39  $\mu$ mol), 500  $\mu$ L of 50 mM sodium cacodylate buffer, pH 6.5 (containing 10 mM EDTA, 20% glycerol and 1 mg/mL bovine serum albumin) and 50  $\mu$ L of GlcNAcT-V (23 mU) were added to a 1.5 mL microcentrifuge tube. The reaction mixture was kept at room temperature. Additional donor (1.9 mg, 2.64  $\mu$ mol) was added after 72 h. After 2 weeks, the reaction was worked up using the same C<sub>18</sub> Sep-Pak procedure that was employed previously for the GlcNAcT-reaction. The dried MeOH residue was chromatographed on an Iatrobead-column eluted with 65:35:3 CHCl<sub>3</sub>-MeOH-water. The pooled product fractions were concentrated, dissolved in water and filtered through a 0.22  $\mu$ m filter before lyophilization. Tetrasaccharide **12** was obtained in a 56% (3.26 mg) isolated yield. Partial <sup>1</sup>H NMR assignments are reported in Table 2. HRESIMS *m/z* 937.3622 (calcd. for C<sub>36</sub>H<sub>61</sub>N<sub>2</sub>O<sub>21</sub>F<sub>3</sub>Na: 937.3617).

*Deprotection of  $\beta$ -D-GlcNAc-(1 $\rightarrow$ 2)-[ $\beta$ -D-GlcNAc-F<sub>3</sub>-(1 $\rightarrow$ 6)]- $\alpha$ -D-Manp-(1 $\rightarrow$ 6)- $\beta$ -D-Glc-OR (R = (CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>) (**12**).*—Deprotection of tetrasaccharide **12** (0.5 mg, 0.55  $\mu$ mol) was successfully accomplished using potassium carbonate in water-MeOH (1:15). The reaction was complete after 1 week at 40 °C. A C<sub>18</sub> Sep-Pak work-up was used to obtain the deprotected tetrasaccharide **13**. Partial <sup>1</sup>H NMR assignments are reported in Table 2. HRESIMS *m/z* 819.3984 (calcd. for C<sub>34</sub>H<sub>63</sub>N<sub>2</sub>O<sub>20</sub>: 819.3974).

## Acknowledgements

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# One-Step, Stereocontrolled Synthesis of Glycosyl 1-Phosphates, Uridine-5'-diphosphogalactose, and Uridine-5'-diphosphoglucose from Unprotected Glycosyl Donors

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**Abstract:** The reaction of 2-(1,2-*trans*-glycopyranosyloxy)-3-methoxypyridines (MOP glycosides) with phosphoric acid leads to the corresponding 1,2-*cis*-1-phosphates in good yield and excellent stereoselectivity. 1-Phosphate esters of  $\alpha$ -D-glucopyranose,  $\alpha$ -D-galactopyranose, and 2-azido-2-deoxy- $\alpha$ -D-galactopyranose were thus prepared without recourse to protective groups. In the L-fucose series, the major product was the  $\alpha$ -L-fucosyl 1-phosphate. An alternative method that relies on neighboring group participation allowed the preparation of a protected  $\beta$ -L-fucosyl 1-phosphate. Reaction of unprotected  $\beta$ -D-glucopyranosyloxy and  $\beta$ -D-galactopyranosyloxy MOP donors with uridine diphosphoric acid gave UDP-Glc and UDP-Gal with preponderance of the desired  $\alpha$ -anomeric configuration.

Glycosyl 1-phosphates and glycosyl 5'-nucleotide esters play a vital role in life processes that involve carbohydrates in particular.<sup>1</sup> Thus, glycosyl 1-phosphates are key intermediates in the metabolism of sugars and their transformation to nucleotides. These in turn are nature's reagents in the enzyme-mediated transfer of a sugar unit (as a glycosyl donor) to an acceptor molecule in the general synthesis of simple and complex glycosides, including oligosaccharides, glycoproteins, and related macromolecules.<sup>2</sup> Unlike protein biosynthesis, the assembly of oligomeric sugars is done through the action of specific glycosyltransferases in the presence of nucleotide 5'-phosphates, hence their paramount importance.

**Glycosyl 1-Phosphates.** The biosynthesis of glycosyl 1-phosphates involves glycosylkinases and ATP. Whitesides and co-workers<sup>2</sup> have reported on practical methods for the enzyme-mediated synthesis of a number of glycosyl 1-phosphates using crude enzyme extracts. For example,  $\alpha$ -D-galactosyl 1-phosphate ( $\alpha$ -D-Gal-1-P) barium salt and  $\alpha$ -D-galactosamine 1-phosphate ( $\alpha$ -GalN-1-P) barium salt could be obtained in a purity of 85–80% and yields of 74–50%, respectively. The 1-phosphates of D-glucose, D-galactose, D-galactosamine, and L-fucose are commercially available at a cost of dollars per milligram depending on the sugar.

The chemical synthesis of glycosyl 1-phosphates<sup>3</sup> has relied on multistep procedures in which an *O*-acetylated glycosyl halide is condensed with a salt of phosphoric acid or its ester, followed by de-esterification. For example, treatment of tetra-*O*-acetyl- $\alpha$ -D-glycopyranosyl bromide with trisilver phosphate or silver diphenyl phosphate in refluxing benzene gives, after a tedious purification protocol,  $\alpha$ -D-glucosyl 1-phosphate ( $\alpha$ -D-

Glc-1-P) as the barium salt.<sup>4</sup> When silver dibenzyl phosphate is used, the product is the  $\beta$ -phosphate barium salt.<sup>5</sup> Although a number of aldosyl 1-phosphates could be synthesized by these and related procedures,<sup>6</sup> the synthesis of  $\beta$ -L-fucosyl 1-phosphate ( $\beta$ -L-Fuc-1-P) has required particular attention. Acetate, benzoate, and *O*-benzyl protective groups have been choice derivatives in the reaction of the corresponding *O*-protected fucosyl halides with phosphate nucleophiles. Problems associated with reproducibility and overall efficiency have been independently addressed by Hindsgaul,<sup>6</sup> Ohlein,<sup>7</sup> and van Boom.<sup>8</sup> Despite improvements, the synthesis of  $\beta$ -L-Fuc-1-P still involves four to five chemical steps from L-fucose.<sup>9</sup> In fact, the natures of *O*-protecting groups and the anomeric leaving group play key roles in the stereoselectivity of phosphorylation.<sup>10,11</sup> The benzoate esters have been reported<sup>10</sup> to provide less of the unwanted anomeric phosphates and to confer some stability compared to acetates for example. Unlike other aldosyl 1-phosphates derivatives, those derived from  $\beta$ -L-fucose must be stored at low temperature and they have a propensity to anomerize to the  $\alpha$ -phosphate triesters at room temperature.<sup>6–10</sup>

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**Glycosyl 5'-Nucleotide Esters: UDP-Gal and UDP-Glc.** The biosynthesis of UDP-Gal can take place via more than one pathway.<sup>12</sup> In the most common protocol, the enzyme UDP-galactopyrophosphorylase catalyses the synthesis of UDP-Gal from  $\alpha$ -D-Gal-1-P and uridine triphosphate (UTP). Another pathway involves the transfer of a uridyl unit from UDP-Glc to  $\alpha$ -D-Gal-1-P with release of Glc-1-P.<sup>13</sup> In some instances, specific isomerasases can transform one sugar residue to another at the UDP-sugar stage as in the biosynthesis of UDP-GalNAc from UDP-GlcNAc and the transformation of UDP-Glc to UDP-Gal.<sup>14</sup> UDP-Glc and UDP-Gal have been isolated from bean seedlings<sup>15</sup> and from yeast<sup>16</sup> by extraction and fractionation techniques. The most practical nonchemical synthesis of UDP-Gal involves the use of UDP-Glc as UMP donor in the presence of  $\alpha$ -D-Gal-1-P and Gal-1-P uridylyltransferase.<sup>2</sup> Thus, UDP-Gal could be isolated as the disodium salt in 43% yield on a 2.5-g scale.

The chemical syntheses of glycosyl 5'-nucleotides have relied mostly on the condensation of glycosyl 1-phosphates with appropriate 5'-nucleotide derivatives.<sup>17</sup> Thus, the ready availability of glycosyl 1-phosphates can be appreciated in this context, since most nucleotide 5'-phosphates or diphosphates are easily accessible.<sup>18</sup>

It is only recently that the synthesis of UDP-Glc by a method that does not involve the condensation of  $\alpha$ -D-Glc-1-P with UMP was disclosed.<sup>19</sup> Thus, 2,3,4,6-tetra-O-benzyl- $\alpha$ -D-glucopyranosyl bromide was condensed with uridine 5'-diphosphoric acid to give, after deprotection, an anomeric mixture of the corresponding UDP-glucose. The same technique was applicable to the synthesis of UDP-Ara and UDP-Fuc in yields ranging from 10 to 30% and rather modest selectivities ( $\alpha/\beta$ , 1:1 to 3:1). Related anomeric phosphorylations with nucleotide 5'-monophosphates have also been reported utilizing *O*-protected sugar derivatives.<sup>20</sup>

In this paper, we describe the stereocontrolled synthesis of  $\alpha$ -1-phosphate esters of D-glucose, D-galactose, L-fucose, and 2-amino-2-deoxygalactose, adopting a method that involves a novel one-step synthesis from unprotected glycosyl donors. The same method is also applicable to the synthesis of UDP-Gal and UDP-Glc. A synthesis of  $\beta$ -L-Fuc-1-P is also described.

## Results and Discussion

We have previously reported on a practical synthesis of *O*-glycosides utilizing 2-( $\beta$ -D-glycosyloxy)-3-methoxypyridine (MOP) donors (Scheme 1).<sup>21</sup> One of the major attributes of the method is the feasibility of stereocontrolled glycoside synthesis without the need for protective groups, reminiscent of enzyme-mediated reactions. In general, 1,2-trans-MOP glycosides with

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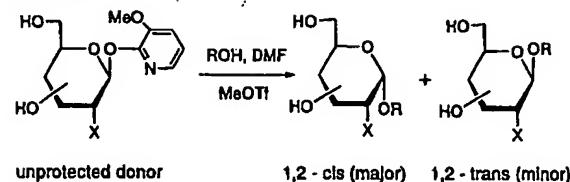
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Scheme 1



hydroxy or azido groups at C-2 in the D-glucos, D-galacto, 2-azido-2-deoxy-D-galacto series led to the corresponding 1,2-cis-*O*-glycosides as preponderant isomers, provided the acceptor (alcohol) component was used in excess (5 equiv or more). Utilizing partially *O*-esterified MOP glycosides as acceptors resulted in the synthesis of di- or oligosaccharides with a latent cleavable MOP group at the new reducing end. De-*O*-esterification and iteration of the process provided a protocol for the assembly of small quantities of certain oligosaccharides.<sup>21</sup>

The utility of MOP and the related 2-pyridylthiocarbonate leaving groups in *O*-protected sugar glycosides as donors in the synthesis of 1,2-cis-oligosaccharides has also been reported.<sup>22,23</sup> Utilizing participating ester groups, in these glycosyl donors led to the corresponding 1,2-trans-glycosides in excellent yields. In the case of *O*-protected glycosyl donors, only a slight excess of acceptor alcohol was used.

In the present study, we sought to develop a one-step stereocontrolled synthesis of four biosynthetically relevant glycosyl 1-phosphates with a desired anomeric configuration. Treatment of the readily available D-glucopyranosyloxy MOP donor (1)<sup>21</sup> with a 6 M excess of phosphoric acid in DMF as solvent led to  $\alpha$ -D-Glc-1-P (2), which was isolated as the dicyclohexylammonium salt in 66% yield, identical with material reported by Putman and Hassid<sup>24</sup> (Scheme 2). Application of this simple procedure to glycosyl MOP donors derived from D-galactopyranose (3) and L-fucopyranose (5) gave the corresponding crystalline  $\alpha$ -D- and  $\alpha$ -L-glycosyl 1-phosphates in yields ranging from 60 to 66%. In the case of 2-azido-2-deoxy- $\alpha$ -D-galactopyranosyl MOP (7), phosphorylation was found to proceed more stereoselectively when dibenzyl phosphate was utilized instead of the free acid. The reaction time was considerably longer than in the above-described cases, requiring 2–3 days for completion. The desired  $\alpha$ -1-dibenzyl phosphate triester (8) was isolated in 64% yield as a homogeneous syrup and characterized by <sup>1</sup>H, <sup>13</sup>C, and <sup>31</sup>P NMR spectroscopies and by chemical correlation. Thus, catalytic hydrogenation of 8 in the presence of Pd(OH)<sub>2</sub> on charcoal under 40 psi of hydrogen pressure, followed by lyophilization, gave the expected  $\alpha$ -D-galactosamine 1-phosphate<sup>3</sup> (9) as a white powder in 95% yield.

As in the case of oligosaccharide synthesis with unprotected 1,2-trans-glycosyloxy MOP donors,<sup>21</sup> the major isolable products were the 1,2-cis-phosphates. We assume that the reaction proceeds via an S<sub>N</sub>2-like mechanism in which a protonated 3-methoxy-2-pyridyloxy moiety is a leaving group. Whether an intermolecular or an intramolecular reaction is involved in the case of phosphoric acid is not known at this time (Scheme 3). Anomerization of the MOP group was not detected under the conditions of the reaction, which corroborates the “inversion” mechanism.

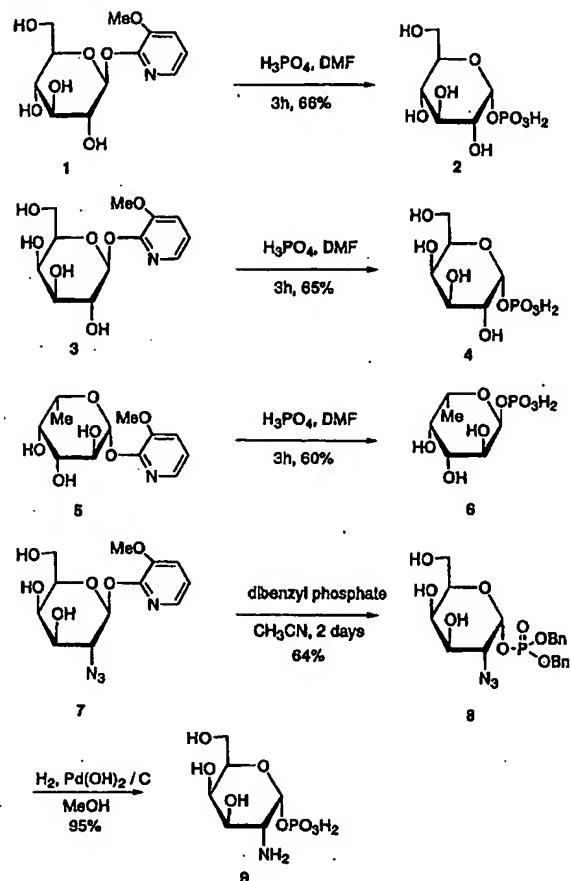
The longer reaction time with 2-azido-2-deoxy- $\beta$ -D-galactopyranosyloxy MOP (7) is not surprising in view of the presence

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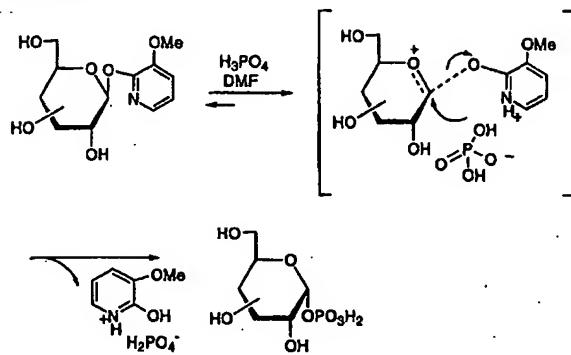
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Scheme 2



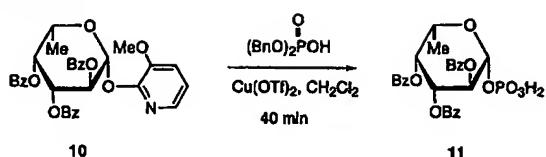
Scheme 3



of the electron-withdrawing azido group at C-2 which makes the pyridyl moiety less prone to protonation.<sup>21</sup> The same behavior was observed in the formation of *O*-glycosides, and the use of triflic acid as an activator was necessary.<sup>21</sup>

As noted above, the direct phosphorylation of a  $\beta$ -L-fucopyranosyl MOP donor led exclusively to  $\alpha$ -L-fucosyl 1-phosphate (6) (Scheme 2). Unfortunately, many attempts to obtain the nucleotide precursor  $\beta$ -L-fucosyl 1-phosphate from an unprotected MOP donor always led to the anomERICALLY more stable  $\alpha$ -phosphate as the major product. We therefore explored the utility of 2,3,4-tri-*O*-benzoyl- $\beta$ -L-fucopyranosyl MOP (10) as a glycosyl donor. In the presence of 0.8 equiv of copper triflate, added portionwise, and a slight excess of dibenzylphos-

Scheme 4



phoric acid, we obtained the desired  $\beta$ -fucosyl phosphate ester 11 in 51% yield as a spectroscopically pure compound (Scheme 4). Minor quantities of the unwanted  $\alpha$ -phosphate (<10%) and starting donor (<10%) could be easily separated by chromatography. The portionwise addition of the catalyst appeared to be an important factor in securing high 1,2-*trans* selectivity. As previously mentioned, and following the initial observations of Whitesides,<sup>10</sup> the synthesis of 11 has been accomplished from the anomeric bromide,<sup>25</sup> thioether,<sup>8</sup> and trichloroacetimidate derivatives.<sup>11</sup>

On the other hand, a recent careful study of the reaction of 2,3,4-tri-*O*-acetyl- $\alpha$ -L-fucopyranosyl bromide with dibenzylphosphoric acid in the presence of 3- $\text{\AA}$  molecular sieves and silver carbonate by Baisch and Ohlein<sup>7</sup> has produced a protocol to prepare the *O*-acetylated  $\beta$ -L-fucosyl 1-phosphate triester on a 800-g scale. This contrasts previous difficulties in working with acetate esters<sup>10</sup> that have proved to be unstable and problematic in isolation and storage.

The MOP technology for anomeric activation has proved its versatility in the above cited examples of direct anomeric phosphorylations with excellent stereocontrol as dictated by the presence or absence of protective groups in the glycosyl donor.

In view of the successful stereoselective phosphorylations of glycosyl MOP derivatives to afford the corresponding anomeric 1-phosphates shown in Schemes 2 and 4, we investigated the analogous 5'-uridyldiphosphorylation reaction. Thus, condensation of the commercially available uridine 5'-diphosphate (UDP) with a glycosyloxy MOP donor in an appropriate solvent was expected to lead to the corresponding UDP-glycose in one step and without recourse to protective groups. Since UDP-Glc and UDP-Gal are biologically relevant nucleotides, we focused on their chemistry in this phase of our work.

In the case of phosphorylations, the acidity and nucleophilicity of phosphoric acid were sufficient to activate the MOP group in an  $\text{S}_{\text{N}}2$ -like reaction to afford the  $\alpha$ -glycosyl 1-phosphates from  $\beta$ -MOP glycosides. It was therefore of interest to see if UDP free acid would be capable of mimicking the reactivity observed in the phosphorylations. Issues related to the generation of UDP free acid<sup>27</sup> and its stability under the conditions of the reaction in DMF as solvent were of concern.

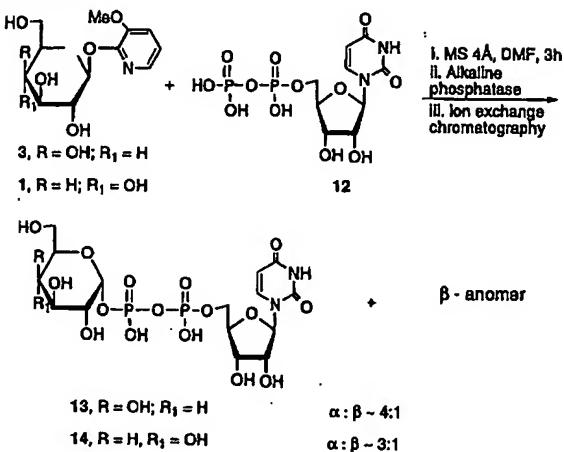
In the event, UDP free acid, prepared from the commercially available trisodium salt 12 by treatment with a cation-exchange resin in DMF, was treated with  $\beta$ -D-galactopyranosyloxy MOP (3) in the presence of powdered 4- $\text{\AA}$  molecular sieves (Scheme 5). After a reaction time of 3 h, the donor was completely consumed with formation of the desired nucleotide contaminated with residual UDP, which was easily separated off by treatment with alkaline phosphatase and its conversion to uridine. Purification of the mixture by ion exchange chromatography gave UDP-Gal (13) as a white powder in 60% yield. Spectroscopic analysis ( $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{31}\text{P}$  NMR) indicated an  $\alpha/\beta$  ratio

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Scheme 5



of 4:1 in favor of the desired nucleotide anomer. UDP-Glc (14) was obtained in a similar manner in ~55% yield and an  $\alpha/\beta$  ratio of 3:1. As reported by Hindsgaul,<sup>19</sup> the presence of the  $\beta$ -isomer does not affect the catalytic activity of glycosyltransferases in enzymatic oligosaccharide synthesis.

In conclusion, we have demonstrated that unprotected 2-( $\beta$ -glycosyloxy)-3-methoxypyridyl donors are versatile intermediates for the one-step stereocontrolled synthesis of  $\alpha$ -1-phosphates of D-glucose, D-galactose, L-fucose, and 2-amino-2-deoxy-D-galactose (via the corresponding 2-azido precursor). The same donors are also capable of transferring glucopyranosyl and galactopyranosyl units to UDP-free acid to afford the corresponding uridine 5'-diphospho sugars in one step. The synthesis of  $\beta$ -L-fucosyl 1-phosphate was achieved through the intermediacy of the *O*-benzoate ester.

The reactions are reminiscent of enzyme-catalyzed syntheses, in that no protection of the hydroxy groups in the glycosyl donor is needed. Future work will focus on extension to nucleotides of other biologically relevant sugars and to further improve anomeric stereocontrol.

## Experimental Section

**General Methods.** Ion-exchange resin Dowex 1×2-200 (chloride form) and 50W×8-100 (H<sup>+</sup> form) were purchased from Aldrich. Uridine 5'-diphosphate (UDP) sodium salt was from Sigma. Ion-exchange resin chromatography was performed on a Bio-Rad Econo system. Alkaline phosphatase (from calf intestine) was purchased from Boehringer Mannheim. <sup>1</sup>H NMR spectra were recorded on 400- or 300-MHz spectrometers with D<sub>2</sub>O ( $\delta$  = 4.81 ppm) or CDCl<sub>3</sub> ( $\delta$  = 7.26 ppm) as an internal reference. <sup>13</sup>C NMR and <sup>31</sup>P NMR spectra were recorded at 100.6 and 162.0 MHz, respectively. Mass spectra were determined by fast atom bombardment (FAB) or electrospray ionization (ESI) techniques. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter at ambient temperature. Melting points measured are uncorrected.

**General Procedure for Preparation of Glycosyl 1-Phosphates:** To a solution of crystalline phosphoric acid (250 mg, 2.5 mmol) in anhydrous DMF (1 mL) was added the glucopyranosyloxy MOP<sup>21</sup> (0.35 mmol). The reaction mixture was stirred at room temperature for 3 h, then neutralized with saturated barium hydroxide. The precipitated barium phosphate was removed by centrifugation and washed with water. The supernatant and washings were combined and concentrated. The residue was dissolved in a small amount of water. Ethanol was added to precipitate the glycosyl 1-phosphate barium salt. The precipitate was collected by centrifugation, and the crude precipitate was reprecipitated with water-ethanol. A solution of glycosyl 1-phosphate barium salt in water (2 mL) was acidified with Amberlite IR-

120 (H<sup>+</sup>), filtered, neutralized with 1 M cyclohexylamine in ethanol, and then concentrated. Precipitation of the residue with water-ethanol gave the glycosyl 1-phosphate bis(cyclohexylammonium) salt in 60–66% yield.

**$\alpha$ -D-Glucopyranosyl 1-Phosphate Bis(cyclohexylammonium) Salt (2).**<sup>24</sup> According to the general procedure described above, 2 was obtained in 66% yield: mp 164–168 °C;  $[\alpha]_D^{20} +64$  (*c* 2.0, H<sub>2</sub>O); reported,<sup>24</sup> mp 163–169 °C;  $[\alpha]_D^{20} +64.0$  (H<sub>2</sub>O); <sup>1</sup>H NMR (D<sub>2</sub>O, 400 MHz)  $\delta$  5.45 (dd, *J* = 7.3, 3.4 Hz, 1H, H-1), 3.93 (ddd, *J* = 9.8, 5.6, 2.1 Hz, 1H, H-5), 3.86 (dd, *J* = 12.2, 2.1 Hz, 1H, H-6b), 3.77 (dd, *J* = 9.8, 9.8 Hz, 1H, H-3), 3.72 (dd, *J* = 12.2, 5.6 Hz, 1H, H-6a), 3.46 (ddd, *J* = 9.8, 3.4, 1.0 Hz, 1H, H-2), 3.38 (dd, 1H, *J* = 9.8, 9.8 Hz, 1H, H-4), 3.14 (m, 2H), 1.01–2.00 (m, 20H); <sup>13</sup>C NMR (D<sub>2</sub>O)  $\delta$  94.26, 73.85, 72.90, 72.67, 70.46, 61.42, 51.02, 31.03, 24.98, 24.49; <sup>31</sup>P NMR (D<sub>2</sub>O)  $\delta$  2.84.

**$\alpha$ -D-Galactopyranosyl 1-Phosphate Bis(cyclohexylammonium) Salt (4).**<sup>24</sup> By following the general procedure described above, 4 was obtained in 65% yield: mp 147–152 °C;  $[\alpha]_D^{20} +76$  (*c* 1.0, H<sub>2</sub>O); reported,<sup>24</sup> mp 147–153 °C;  $[\alpha]_D^{20} +78.5$  (H<sub>2</sub>O); <sup>1</sup>H NMR (D<sub>2</sub>O, 400 MHz)  $\delta$  5.45 (dd, *J* = 7.2, 3.5 Hz, 1H, H-1), 4.13 (dd, *J* = 6.2, 6.2 Hz, 1H, H-5), 3.95 (d, *J* = 3.4 Hz, 1H, H-4), 3.86 (dd, *J* = 10.2, 3.2 Hz, 1H, H-3), 3.66–3.79 (m, 3H, H-2, H-6a and H-6b), 3.16 (m, 2H), 1.04–1.99 (m, 20H); <sup>13</sup>C NMR (D<sub>2</sub>O)  $\delta$  94.84, 71.91, 70.20, 70.09, 69.53, 61.98, 51.05, 31.04, 24.99, 24.50; <sup>31</sup>P NMR (D<sub>2</sub>O)  $\delta$  2.06.

**$\alpha$ -L-Fucopyranosyl 1-Phosphate Bis(cyclohexylammonium) Salt (6).**<sup>24</sup> By following the general procedure described above, 6 was obtained in 60% yield: mp 168–176 °C;  $[\alpha]_D^{20} -76$  (*c* 1.0, H<sub>2</sub>O); reported,<sup>24</sup> mp 147–153 °C;  $[\alpha]_D^{20} -77.8$  (H<sub>2</sub>O); <sup>1</sup>H NMR (D<sub>2</sub>O, 400 MHz)  $\delta$  5.44 (dd, *J* = 6.8, 3.4 Hz, 1H, H-1), 4.28 (q, *J* = 6.6 Hz, 1H, H-5), 3.93 (dd, *J* = 10.2, 3.2 Hz, 1H, H-3), 3.82 (d, *J* = 3.2 Hz, H-4), 3.71 (ddd, *J* = 10.2, 3.4, 1.0 Hz, 1H, H-2), 3.16 (m, 2H), 1.21 (d, *J* = 6.6 Hz, 1H, H-6), 1.16–2.01 (m, 20H); <sup>13</sup>C NMR (D<sub>2</sub>O)  $\delta$  94.53, 72.78, 70.52, 69.56, 67.60, 51.06, 31.04, 24.99, 24.50, 16.18; <sup>31</sup>P NMR (D<sub>2</sub>O)  $\delta$  2.62.

**2-Azido-2-deoxy- $\alpha$ -D-galactopyranosyl 1-Dibenzyl Phosphate (8).** To a solution of dibenzyl phosphate (555 mg, 4.96 mmol) in anhydrous acetonitrile (30 mL) at 0 °C was added a solution of 7 (155 mg, 0.496 mmol) in CH<sub>3</sub>CN (6 mL) dropwise. The reaction mixture was warmed to room temperature and stirred for 2–3 days. After the reaction was completed, the mixture was neutralized with pyridine and concentrated to dryness. Column chromatography of the residue on silica gel using EtOAc–MeOH–Et<sub>3</sub>N (90:10:1) as an eluent afforded the desired product 8 as a syrup (148 mg, 64%);  $[\alpha]_D^{20} +34$  (*c* 1.5, MeOH); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 300 MHz)  $\delta$  7.32–7.39 (m, 10H, Ph), 5.79 (dd, *J* = 6.0, 3.2 Hz, 1H, H-1), 5.13 and 5.11 (2 AB, *J* = 11.0 Hz, 4H, CH<sub>2</sub>Ph), 3.94 (m, 1H, H-5), 3.93 (d, *J* = 2.9 Hz, 1H, H-4), 3.87 (dd, *J* = 10.4, 2.9 Hz, 1H, H-3), 3.76 (ddd, *J* = 10.4, 3.2, 3.2 Hz, H-2), 3.67 (d, *J* = 6.6 Hz, 2H, H-6); <sup>13</sup>C NMR (CD<sub>3</sub>OD)  $\delta$  131.36, 131.22, 131.00, 130.83, 130.51, 100.48, 76.68, 72.75, 72.70, 71.39, 71.30, 64.01; <sup>31</sup>P NMR (D<sub>2</sub>O)  $\delta$  1.61. The product was unstable upon storage.

**2-Amino-2-deoxy- $\alpha$ -D-galactopyranosyl 1-Phosphate ( $\alpha$ -GalN-1-P, 9).**<sup>24</sup> To a solution of the preceding compound 8 (60 mg, 0.13 mmol) in MeOH (2 mL) was added Pd(OH)<sub>2</sub> (150 mg, 20 wt % on charcoal). The reaction mixture was stirred under 40 psi H<sub>2</sub> for 2 h, filtered, and concentrated. Lyophilization of the residue afforded the title compound 9 as a white powder (31 mg, 95%);  $[\alpha]_D^{20} +137.7$  (*c* 0.44, H<sub>2</sub>O); <sup>1</sup>H NMR (D<sub>2</sub>O, 400 MHz)  $\delta$  5.75 (dd, *J*<sub>1,2</sub> = 3.3 Hz, *J*<sub>1,3</sub> = 6.7 Hz, 1H, H-1), 4.20 (br t, *J*<sub>1,5</sub> = 6.5 Hz, 1H, H-5), 4.14 (dd, *J*<sub>2,3</sub> = 11.0 Hz, *J*<sub>3,4</sub> = 3.0 Hz, 1H, H-3), 4.08 (br d, *J*<sub>3,4</sub> = 3.0 Hz, 1H, H-4), 3.80 (dd, *J*<sub>gem</sub> = 11.8 Hz, *J*<sub>5,6</sub> = 7.2 Hz, 1H, H-6a), 3.74 (dd, *J*<sub>gem</sub> = 11.8 Hz, *J*<sub>5,6</sub> = 5.4 Hz, 1H, H-6b), 3.56 (dt, *J*<sub>2,1</sub> = 10.9 Hz, *J*<sub>1,2</sub> = *J*<sub>2,3</sub> = 3.2 Hz, 1H, H-2); <sup>13</sup>C NMR (D<sub>2</sub>O)  $\delta$  92.38 (d, *C*1, *J* = 5.0 Hz), 72.42 (C5), 68.76 (C4), 67.07 (C3), 61.63 (C6), 51.85 (d, *J* = 8.1 Hz, C2); <sup>31</sup>P NMR (D<sub>2</sub>O)  $\delta$  0.50.

**2-(2,3,4-Tri-O-benzoyl- $\beta$ -L-fucopyranosyloxy)-3-methoxypyridine (10).** A mixture of 2-( $\beta$ -L-fucopyranosyloxy)-3-methoxypyridine<sup>21</sup> (68 mg, 0.25 mmol), benzoic anhydride (1.02 g, 4.50 mmol), DMAP (138 mg, 1.13 mmol), and pyridine (2 mL) was stirred at room temperature for 2 h. Methanol (1 mL) was added, and the mixture was stirred for 30 min, then diluted with CH<sub>2</sub>Cl<sub>2</sub> (10 mL), washed with saturated NaHCO<sub>3</sub>, H<sub>2</sub>O, and brine, dried (Na<sub>2</sub>SO<sub>4</sub>), and concentrated. The residue was purified by column chromatography using hexanes–

$\text{EtOAc}-\text{Et}_3\text{N}$  (100:50:1) as an eluent to provide the title compound (132 mg, 90%) as a white foam:  $[\alpha]_D -191.6$  ( $c$  0.80,  $\text{CH}_2\text{Cl}_2$ );  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  8.15, 7.88, 7.82 (ddd,  $J = 8.0, 1.5, 1.0$  Hz, each 2H, benzoyl C2-H, C6-H), 7.75 (dd,  $J = 4.9, 1.5$  Hz, 1H, MOP H-4), 7.61 (dddd,  $J = 8.0, 7.8, 1.2, 1.0$  Hz, 1H, Ar-H), 7.49 (dddd,  $J = 8.0, 7.8, 1.2, 1.0$  Hz, 2H, Ar-H), 7.48–7.41 (m, 2H, Ar-H), 7.29, 7.26 (ddd,  $J = 8.0, 7.8, 0.8$  Hz, each 2H, Ar-H), 7.01 (dd,  $J = 7.8, 1.5$  Hz, 1H, MOP H-6), 6.90 (dd,  $J = 7.8, 4.9$  Hz, 1H, MOP H-5), 6.53 (d,  $J_{1,2} = 8.3$  Hz, 1H, H-1), 6.12 (dd,  $J_{2,3} = 10.4$  Hz, 1H, H-2), 5.81 (dd,  $J_{3,4} = 3.5$  Hz =  $J_{4,5} = 1.0$  Hz, 1H, H-4), 5.71 (dd, 1H, H-3), 4.35 (dq,  $J_{5,6} = 6.4$  Hz, 1H, H-5), 3.70 (s, 3H,  $\text{OCH}_3$ ), 1.38 (d, 3H, H-6);  $^{13}\text{C}$  NMR (100.6 MHz,  $\text{CDCl}_3$ )  $\delta$  166.0, 165.6, 165.1 (C=O), 152.0 (MOP C2), 144.2, 136.7, 133.3, 133.1, 132.9, 130.01, 129.99, 129.97, 129.95, 129.72, 129.70, 129.68, 129.66, 129.65, 129.60, 129.59, 129.57, 129.43, 129.18, 128.87, 128.47, 128.45, 128.43, 128.21, 128.19, 128.17, 128.16, 128.14, 128.13, 128.11, 128.10, 128.08, 119.13, 118.82 (aromatic C, CH), 94.55 (C-1), 72.4, 71.0, 70.5, 69.4 (C2, C3, C4, CS), 55.7 ( $\text{OCH}_3$ ), 16.3 (C6); FAB MS ( $m/z$ ) 584 ( $\text{M} + \text{H}$ ) $^+$ ; exact FAB MS calcd for  $\text{C}_{31}\text{H}_{30}\text{NO}_4$  ( $\text{M} + \text{H}$ ) $^+$  584.1920, found 584.1921.

**Dibenzylyl 2,3,4-Tri-O-benzoyl- $\beta$ -L-fucopyranosyl 1-Phosphate (11).** To a mixture of 10 (58.4 mg, 0.10 mmol), dibenzyl phosphate (55.7 mg, 0.20 mmol), crushed 4-Å-molecular sieves (584 mg), and anhydrous  $\text{CH}_2\text{Cl}_2$  (10 mL) was added copper triflate (29 mg, 0.08 mmol) in five portions within 40 min under argon. The reaction was monitored by TLC and neutralized with pyridine. The suspension was filtered through a Celite pad, and the filtrate was concentrated. The residue was subjected to silica gel chromatography using  $\text{CH}_2\text{Cl}_2$ –acetone– $\text{Et}_3\text{N}$  (98:2:1) as an eluent to give the title compound (38 mg, 51%);<sup>25</sup> the  $\alpha$ -anomer (7.4 mg, 10%), and unreacted starting material (6 mg, 10%):  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.12, 7.96, 7.80 (dd,  $J = 8.0, 1.2$  Hz, each 2H, benzoyl C2-H, C6-H), 7.68–6.99 (m, 19H, Ar-H), 5.89 (dd,  $J_{2,3} = 10.4$  Hz,  $J_{1,2} = 8.0$  Hz, 1H, H-2), 5.76 (dd,  $J_{3,4} = 3.2$  Hz,  $J_{4,5} = 1.0$  Hz, 1H, H-4), 5.67 (dd,  $J_{1,2} = 7.2$  Hz, 1H, H-1), 5.57 (dd, 1H, H-3), 5.14, 5.12 (ABM,  $J = 11.8$  Hz, 7.4 Hz, each 1H,  $\text{PhCH}_2$ ), 4.86, 4.77 (ABM,  $J = 11.6, 6.4$  Hz, each 1H,  $\text{PhCH}_2$ ), 4.22 (dq,  $J_{5,6} = 6.4$  Hz, 1H, H-5), 1.35 (d, 1H, H-6);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  165.7, 165.4, 165.2 (C=O), 135.5, 135.4, 135.04, 134.96, 133.45, 133.35, 133.21, 129.89, 129.73, 129.68, 129.03, 128.80, 128.63, 128.53, 128.39, 128.26, 128.21, 127.83, 127.35 (aromatic C, CH), 96.94 (d,  $J_{1,2} = 4.7$  Hz, C-1), 71.7, 70.8, 70.5 (C3, C4, CS), 69.6 (d,  $J_{2,3} = 9.3$  Hz, C-2), 69.5 (d,  $J_{1,2} = 5.6$  Hz,  $\text{PhCH}_2$ ), 69.2 (d,  $J_{1,2} = 5.6$  Hz,  $\text{PhCH}_2$ ), 16.1 (C6);  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ )  $\delta$  -2.41; FAB MS ( $m/z$ ) 737 ( $\text{M} + \text{H}$ ) $^+$ ; exact FAB MS calcd for  $\text{C}_{41}\text{H}_{38}\text{O}_{11}\text{P}$  ( $\text{M} + \text{H}$ ) $^+$  737.2156, found 737.2154.

**Uridine 5'-Diphospho-D-galactose (UDP-Glc, 14).** UDP-Glc was prepared from UDP free acid and GlcOMOP using the same procedure as described above for the preparation of UDP-Gal except that the time of reaction was extended to 6 h. UDP-Glc was obtained as a white powder (~50%,  $\alpha/\beta$  ratio 3:1):  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz)  $\delta$  7.92 (d,  $J = 8.0$  Hz, 1H, H-6’), 5.96 (d,  $J = 8.1$  Hz, 1H, H-5’), 5.94 (d,  $J = 4.2$  Hz, 1H, H-1’), 5.62 (dd,  $J = 7.0, 3.0$  Hz, H-1 of  $\alpha$ -Gal), 4.94 (dd,  $J = 7.4, 7.2$  Hz, H-1 of  $\beta$ -Gal), 4.20 (d,  $J = 2.5$  Hz, H-4), 3.62 (dd,  $J = 9.6, 7.3$  Hz, H-2 of  $\beta$ -Gal);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  166.95, 152.54, 142.33, 103.37, 96.55 (d,  $J = 6.9$  Hz, C1), 89.07, 83.91, 76.56, 74.48, 72.61, 70.36, 70.02, 69.12 (d,  $J = 8.5$  Hz, C2), 65.64 (d,  $J = 5.8$  Hz, C5’), 61.91, 61.71;  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  -10.49 and -12.03; ESI MS  $m/z$  633 ( $\text{M} + \text{Na}$ ) $^+$ , 611 ( $\text{M} + \text{H}$ ) $^+$ . The ammonium salt of 13 was not suitable for MS analysis.

**Uridine 5'-Diphospho-D-glucose (UDP-Glc, 14).** UDP-Glc was prepared from UDP free acid and GlcOMOP using the same procedure as described above for the preparation of UDP-Gal except that the time of reaction was extended to 6 h. UDP-Glc was obtained as a white powder (~50%,  $\alpha/\beta$  ratio 3:1):  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz)  $\delta$  7.92 (d,  $J = 8.0$  Hz, 1H, H-6’), 5.99 (d, 1H,  $J = 3.5$  Hz, 1H, H-1’), 5.94 (d,  $J = 8.0$  Hz, 1H, H-5’), 5.60 (dd,  $J = 7.3, 3.3$  Hz, H-1 of  $\alpha$ -Glc), 5.01 (dd,  $J = 8.0, 7.9$  Hz, H-1 of  $\beta$ -Gal), 3.78 (dd,  $J = 9.8, 9.8$  Hz, H-3), 3.53 (ddd,  $J = 9.8, 3.3, 3.0$  Hz, H-2 of  $\alpha$ -Glc), 3.47 (dd,  $J = 9.8, 9.8$  Hz, H-4), 3.39 (ddd,  $J = 9.8, 8.0, 3.0$  Hz, H-2 of  $\beta$ -Gal);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  166.94, 152.52, 142.28, 103.39, 103.33, 98.55 (C1 of  $\beta$ -Glc), 96.27 (d,  $J = 6.7$  Hz, C1 of  $\alpha$ -Glc), 89.02, 88.92, 83.94, 83.85, 77.17, 74.44, 74.25, 73.49, 70.40, 70.30, 70.10, 69.82, 65.62, 65.56, 61.46, 60.92;  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  -10.62 and -12.26; ESI MS  $m/z$  633 ( $\text{M} + \text{Na}$ ) $^+$ , 611 ( $\text{M} + \text{H}$ ) $^+$ .

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**Supporting Information Available:**  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{31}\text{P}$  NMR spectra for all compounds (24 pages, print/PDF). See any current masthead page for ordering information and Internet access instructions.

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## INHIBITORS OF THE BACTERIAL CELL WALL BIOSYNTHESIS ENZYME MUR D

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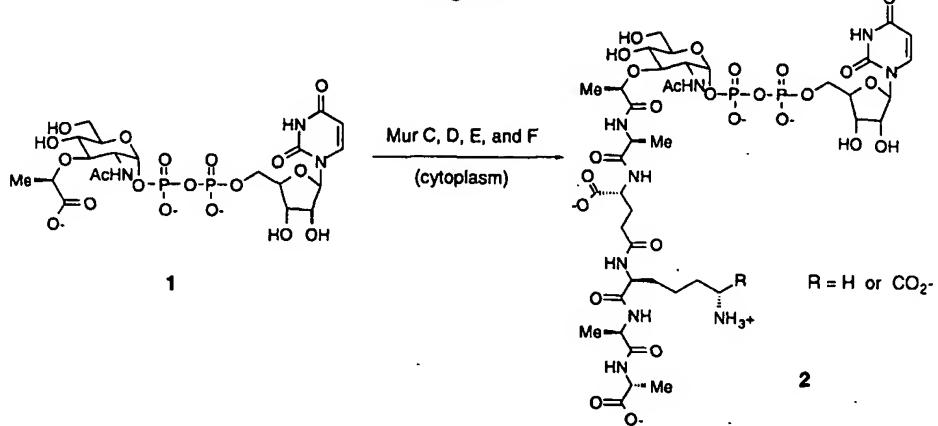
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**Abstract:** A series of transition-state analog inhibitors of the D-glutamic acid-adding enzyme (MurD) of bacterial peptidoglycan biosynthesis has been synthesized and evaluated for inhibition of the *E. coli* enzyme.

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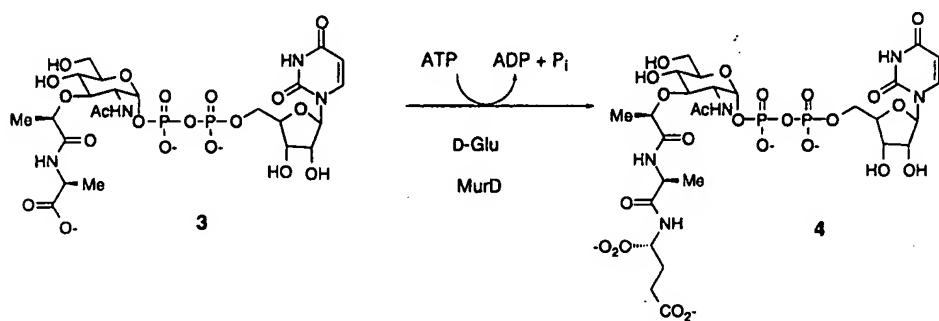
The bacterial cell wall peptidoglycan layer consists of alternating *N*-acetyl muramic acid (MurNAc) and *N*-acetyl glucosamine units that are crosslinked through pentapeptide chains. The disruption of this structure leads to cell lysis; peptidoglycan biosynthesis is therefore an essential pathway and an important target for antibiotics research.<sup>1</sup> The final cytoplasmic precursor for peptidoglycan biosynthesis is the uridine diphosphate-MurNAc-pentapeptide **2** (Figure 1). This compound is synthesized from UDP-MurNAc **1** by four amino acid ligases, Mur C, D, E, and F, that add L-Ala, D-Glu, *meso*-DAP (or L-Lys in some Gram-positive bacteria), and D-Ala-D-Ala, respectively. MurC through F are believed to function in a similar manner to the ATP-dependent amide-forming enzymes glutamine synthetase,<sup>2</sup> glutathione synthetase,<sup>3</sup> and D-Ala-D-Ala ligase.<sup>4</sup> These latter enzymes each catalyze the formation of an acyl phosphate that is attacked by the incoming amino acid to form a tetrahedral species that collapses to the amide product. Recent mechanistic studies on MurC<sup>5</sup> and MurD<sup>6</sup> support the hypothesis that the Mur ligases also proceed by the acyl phosphate mechanism. This information is especially useful in designing potential inhibitors of the Mur enzymes, as it is known that phosphinates act as slow-binding inhibitors of glutamine synthetase<sup>7</sup>, glutathione synthetase<sup>8</sup> and D-Ala-D-Ala ligase.<sup>9,10</sup>

Figure 1



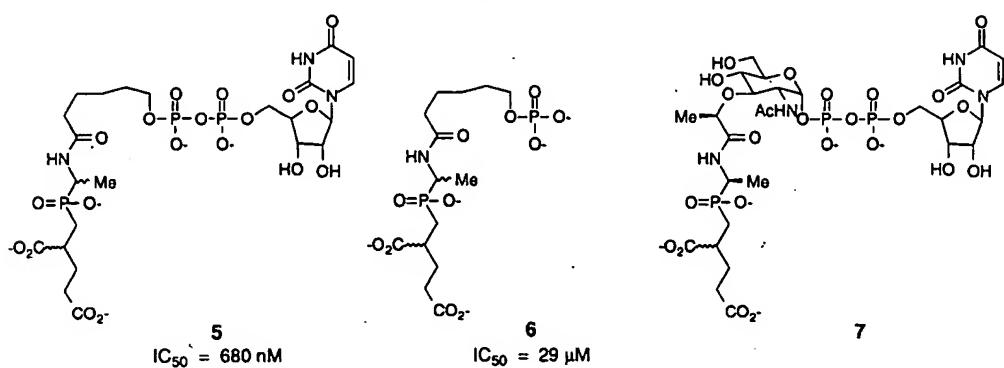
MurD, the second enzyme in the series of peptidoglycan biosynthesis ligases, catalyzes the addition of D-glutamic acid to UDP-MurNAc-L-alanine **3** to afford UDP-MurNAc-L-Ala-D-Glu **4** (Figure 2). Recently, Tanner and coworkers reported the first effective inhibitors of MurD (Compounds **5** and **6**, Figure 3).<sup>11</sup> Tanner's transition-state analog inhibitors **5** and **6** feature a phosphinate transition-state analog at the center of amide bond formation and a methylene chain as a replacement for the *N*-acetyl muramic acid moiety. Compound **5** was found to be a good inhibitor of MurD with an  $IC_{50}$  value of 680 nM, while phosphate **6** was a weaker inhibitor with  $IC_{50}$  of 29  $\mu$ M.<sup>11</sup>

Figure 2



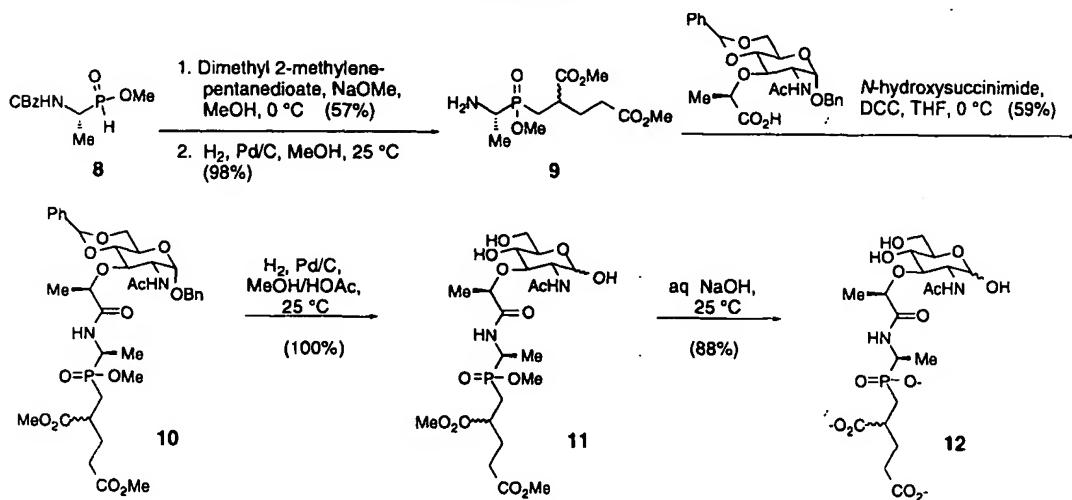
We were interested in preparing MurD inhibitors that would be more potent than those that had already been reported. We hypothesized that the *N*-acetyl muramic acid structure could be an important contributor to potency and we therefore chose **7** as our initial MurD inhibitor target molecule. In our first-generation inhibitor **7**, we have retained the proven phosphinate transition-state mimic design. However, we have incorporated the carbohydrate moiety and controlled the stereochemical configuration of the  $\alpha$ -amino phosphinate. Here we report the synthesis and biological evaluation of **7** and its synthetic precursors.

Figure 3



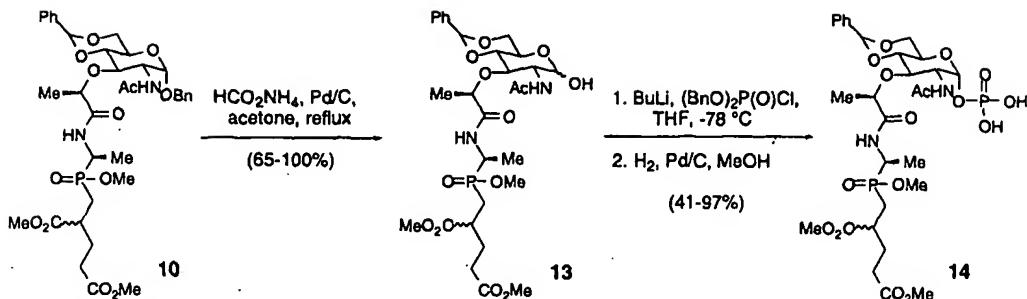
Our starting material, the known methyl phosphinate **8**, was synthesized in six steps according to the procedure of Baylis.<sup>12</sup> This included a resolution of the free phosphinous acid by recrystallization of its salt with *R*- $\alpha$ -methylbenzylamine. Following Tanner's precedent, compound **8** was treated with sodium methoxide and dimethyl 2-methylene pentanedioate<sup>13</sup> to afford the fully protected dipeptide isostere as a mixture of four diastereomers (Scheme 1). The CBz group was removed via hydrogenolysis in preparation for formation of the amide bond to link the phosphinate and sugar fragments. The amine **9** was added to benzyl-*N*-acetyl-4,6-*O*-benzylidene muramic acid<sup>14</sup> activated by *N*-hydroxysuccinimide/DCC. The amide product **10** was isolated in 59% yield and was also found to be a mixture of four diastereomers by <sup>31</sup>P NMR. Removal of the benzyl and benzylidene protecting groups from amide **10** proceeded quantitatively and was followed by saponification of the triol **11** to afford the phosphinate **12** as its trisodium salt. This compound containing a free anomeric hydroxyl group was of particular interest to us in establishing our preliminary SAR for the inhibition of MurD.

Scheme 1



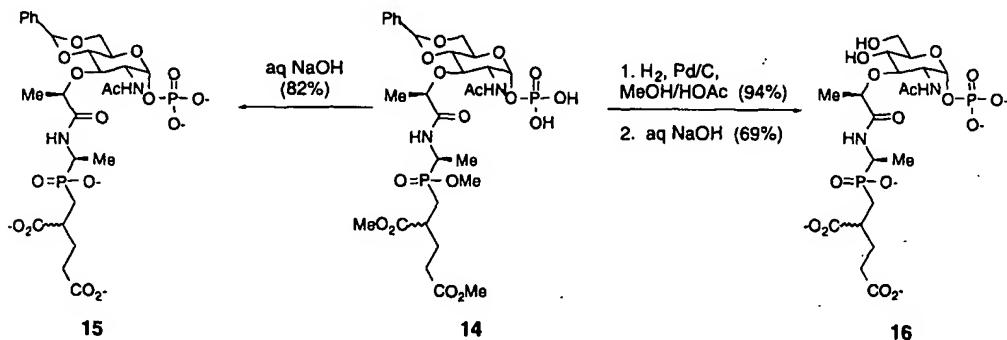
We then turned our attention to the phosphorylation of the anomeric position. All of our attempts to phosphorylate triol **11** were unsuccessful, despite literature precedent for the phosphorylation of unprotected glucoses.<sup>15</sup> It was therefore necessary to return to the fully protected derivative **10** and deprotect the muramic acid in a stepwise manner (Scheme 2). The benzyl group was selectively removed from amide **10** by catalytic transfer hydrogenation using ammonium formate as the hydrogen donor<sup>16</sup> to afford the anomeric alcohol **13**. The alcohol **13** was phosphorylated<sup>17</sup> by treatment with *n*-BuLi and dibenzylchlorophosphate to afford the unstable muramic acid dibenzyl phosphate. Therefore, immediately upon consumption of **13**, the reaction mixture was allowed to warm to room temperature and stir under an atmosphere of H<sub>2</sub> in the presence of Pd/C catalyst. This phosphorylation procedure selectively produced the 1- $\alpha$ -phosphate **14** in 97% yield. Once again, this compound was a mixture of four diastereomers due to mixtures at the phosphinate chiral center and at the carbon chiral center located at the  $\beta$ -position to the phosphinate.

Scheme 2



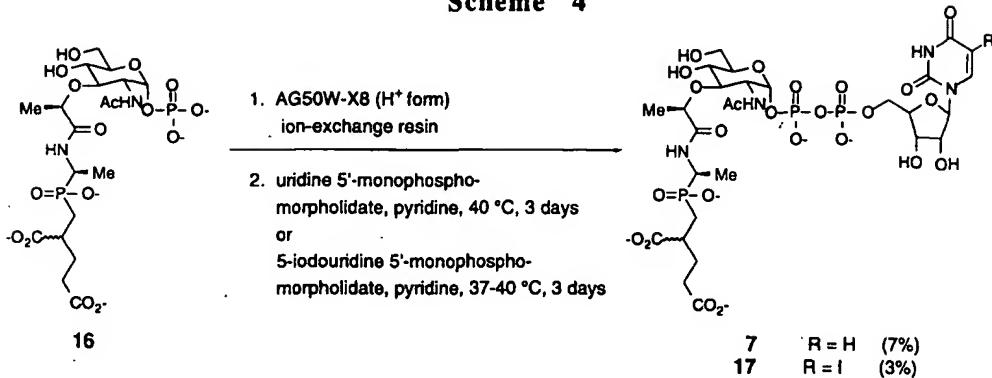
In order to reach our desired target, the benzylidene group was removed from 14 via hydrogenolysis in the presence of acetic acid (Scheme 3). The resulting diol was saponified to afford phosphate 16 as its pentasodium salt. At this stage, only formation of the pyrophosphate bond was required to complete the synthesis of the fully elaborated inhibitor 7. However, in order to probe the effect of functionality at the 4,6-position on potency, the substituted phosphoric acid 14 was saponified to yield the benzylidene-protected pentasodium salt 15.

Scheme 3



In the final step of the synthesis, the pyrophosphate bond was formed via Khorana's standard morpholidate-activated coupling conditions.<sup>18</sup> The free acid form of phosphate 16 was allowed to react with uridine 5'-monophosphomorpholidate to afford the desired diphosphate 7 (Scheme 4) as a mixture of two diastereomers. In addition, the 5-iodo analog 17 was synthesized in a similar manner by allowing 16 to react with 5-iodo-5'-monophosphomorpholidate. Our isolated yields were poor (7%, 3%) and we were not able to improve them by use of 1*H*-tetrazole as a catalyst.<sup>19</sup>

Scheme 4



We tested compounds **12**, **15**, **16**, **17**, and **7** for inhibition against the MurD enzyme isolated from *E. coli*. The reaction catalyzed by MurD was followed by the formation of radiolabelled UDP-MurNAc-L-Ala-D-Glu from radiolabelled D-[2,3,4-<sup>3</sup>H]-glutamic acid (25  $\mu$ M) and UDP-MurNAc-L-Ala (25  $\mu$ M). The  $IC_{50}$ s were determined in the presence of 850 nM enzyme for compound **12** and 1 nM enzyme for all other compounds. Our results are summarized in Table 1; Tanner's data<sup>11</sup> have been included for comparison.

Table 1

Compound	<b>12</b>	<b>15</b>	<b>16</b>	<b>17, <math>R = I</math></b>	<b>7, <math>R = H</math></b>
$IC_{50}$ (nM)	782,000	455	20	4	<1
Compound	<b>6</b>	<b>5</b>			
$IC_{50}$ (nM)	29,000 <sup>11</sup>	680 <sup>11</sup>			

We conclude that the incorporation of muramic acid and the resolution of one stereocenter significantly increase potency, perhaps by more than three orders of magnitude. This increase in potency is evident when Tanner's terminal phosphate inhibitor **6** (29  $\mu$ M) is compared to our terminal phosphate **16** (20 nM). The  $IC_{50}$  of our uridine-containing inhibitor **7** (<1 nM) lies below the sensitivity of the enzyme assay; we see that it is at least approaching a value three orders of magnitude below the  $IC_{50}$  of Tanner's analogous compound **5** (680 nM). Within our series, the phosphate group is critical (**16**, 20 nM) as its presence adds more than four orders of magnitude in potency to the terminal hydroxy compound **12** (782  $\mu$ M). It is interesting to note that the 4,6-*O*-benzylidene group is reasonably well tolerated, with only a 20-fold difference in potency observed between compounds **15** and **16**.

**Acknowledgments** We wish to thank Mrs. Tracey Klatt for running LC-ESIMS, Dr. Barbara Leiting and Mrs. Kelly Ann Pryor for synthesizing the MurD enzyme, and Dr. Milton Hammond for helpful discussions.

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data, mp,  $\lambda_{\text{max}}$ , and  $\epsilon$  for 6 except for 6e were reported previously.<sup>1</sup> 1-(*tert*-Butylamino)-2,4-dinitronaphthalene (6e): mp 115–115.5 °C;  $\lambda_{\text{max}}$  408 nm (11600 [MeOH]);  $^1\text{H}$  NMR (DMSO- $d_6$ )  $\delta$  1.33 [s, 9 H, C(CH<sub>3</sub>)<sub>3</sub>], 7.97 (br s, 1 H, NH, overlapped with H<sup>6,7</sup>), 8.03 (qui, 2 H, H<sup>6,7</sup>, partially overlapped with NH), 8.70 (m, 2 H, H<sup>6,8</sup>, overlapped with each other), 9.03 (s, 1 H, H<sup>3</sup>), in which the suffix on hydrogen represents the positional number of a naphthalene moiety.

**Determination of Products.** The typical procedure for determination of the reaction products is described for the reaction of 6d with methylamine. The 10-mL DMSO solution containing 5 mmol (0.137 g) of 6d and 3 equiv of methylamine (40% solution) was stirred for the prescribed time at the prescribed temperature,

and then the mixture was poured into 200 mL of water, acidified with the equiv of HCl based on the methylamine added, extracted with 200 mL of benzene three times, and dried over anhydrous MgSO<sub>4</sub>. After the mixture was filtered, the benzene layer was subjected to HPLC (Shimazu LC-6A, silica gel, hexane-2-propanol (20:1 v/v).

Registry No. 6a, 39139-78-1; 6b, 27210-67-9; 6c, 124855-05-6; 6d, 118209-15-7; 6f, 118062-03-4; 6g, 124855-07-8; 6h, 92859-08-0; 6i, 68105-52-2; 9 (R = Pr), 124855-08-9; MeNH<sub>2</sub>, 74-89-5; EtNH<sub>2</sub>, 75-04-7; PrNH<sub>2</sub>, 107-10-8; i-PrNH<sub>2</sub>, 75-31-0; t-BuNH<sub>2</sub>, 75-64-9; BuNH<sub>2</sub>, 109-73-9; p-MeC<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>, 106-49-0; p-NO<sub>2</sub>C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>, 100-01-6; DMSO, 67-68-5.

## Convenient Syntheses of Cytidine 5'-Triphosphate, Guanosine 5'-Triphosphate, and Uridine 5'-Triphosphate and Their Use in the Preparation of UDP-glucose, UDP-glucuronic Acid, and GDP-mannose

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This paper compares enzymatic and chemical methods for the synthesis of cytidine 5'-triphosphate, guanosine 5'-triphosphate, and uridine 5'-triphosphate from the corresponding nucleoside monophosphates on scales of ~10 g. These nucleoside triphosphates are important as intermediates in Leloir pathway biosyntheses of complex carbohydrates; the nucleoside monophosphates are readily available commercially. The best route to CTP is based on phosphorylation of CMP using adenylate kinase (EC 2.7.4.3); the route to GTP involves phosphorylation of GMP using guanylate kinase (EC 2.7.4.8); chemical deamination of CTP (prepared enzymatically from CMP) is the best synthesis of UTP. For the 10–200-mmol-scale reactions described in this paper, it is more convenient to prepare phosphoenolpyruvate (PEP), used in the enzymatic preparations, from D-(–)-3-phosphoglyceric acid (3-PGA) in the reaction mixture rather than to synthesize PEP in a separate chemical step. The in situ conversion of 3-PGA to PEP requires the coupled action of phosphoglycerate mutase (EC 2.7.5.3) and enolase (EC 4.2.1.11). The enzyme-catalyzed syntheses of uridine 5'-diphosphoglucose (UDP-Glc), uridine 5'-diphosphoglucuronic acid (UDP-GlcUA), and guanosine 5'-diphosphomannose (GDP-Man) illustrate the use of the nucleoside triphosphates.

### Introduction

As part of a broad program<sup>3</sup> to develop synthetic techniques based on glycosyl transferases for the preparation of glycoproteins, glycolipids, and proteoglycans,<sup>4</sup> we wished to develop convenient routes to cytidine 5'-triphosphate (CTP), guanosine 5'-triphosphate (GTP), and uridine 5'-triphosphate (UTP). Enzyme-catalyzed reactions of these three compounds with monosaccharides are central reactions in the biosynthesis of the nucleoside phosphate sugars required by glycosyl transferases in mammalian biochemistry (CMP-NeuAc, GDP-Fuc, GDP-Man, UDP-Gal, UDP-GalNAc, UDP-Glc, UDP-GlcNAc, UDP-GlcUA, and UDP-Xyl).<sup>5</sup>

An important issue in planning synthetic tactics concerns the method of synthesizing the NTPs and nucleoside phosphate sugars for use in enzyme-catalyzed reactions: should they be synthesized independently and used as stoichiometric reagents (in which case chemical, enzymatic or fermentation syntheses would all, in principle, be acceptable) or should they be generated and used in situ (in which case only enzymatic syntheses would be acceptable)? We have decided initially to develop synthetic methods

that generate the NTPs and nucleoside phosphate sugars as stoichiometric reagents, rather than relying on their generation in situ, for five reasons. First, this type of approach is the most practical. Developing complex systems of coupled enzymes is difficult. If the syntheses of the NTPs and nucleoside phosphate sugars can be developed and optimized separately, the final systems are simpler. Second, this approach has greater generality. If convenient routes to all of the NTPs and nucleoside phosphate sugars can be developed, these compounds are then available for the full range of oligo- and polysaccharide syntheses. Third, this approach is the most flexible. By conducting syntheses of these compounds separately, it is possible to use whatever synthetic method works best for each, without concern for the compatibility of these methods. Fourth, separating syntheses of the nucleoside phosphate sugars from the steps involving use of these compounds in forming glycosidic bonds permits the latter reactions to be conducted in a way that optimizes the use of the glycosyl transferases (normally the most difficult enzymes to obtain and use).<sup>3</sup> Finally, this approach is more likely to be successful for the synthesis of unnatural compounds, where analogues of the natural reactants may have to be synthesized chemically.

CTP, GTP, and UTP are all available from commercial sources, but their cost precludes their use in multigram-scale reactions. We do not discuss in detail the synthesis of adenosine 5'-triphosphate (ATP) here because it is relatively inexpensive compared with other NTPs,<sup>6</sup> and

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Table I. Scale and Yields for Enzymatic Synthesis of Nucleoside Triphosphates

NTP	enzyme <sup>a</sup>	phosphoryl donor <sup>b</sup>	amount of NTP, g (yield, %)	reaction time (days)
CTP	AdK	3-PGA	145 (92)	3
GTP	GK	3-PGA	12 (82)	3
	GK	PEP	12 (88)	3
	AdK (Mn <sup>2+</sup> )	PEP		3, no reactn
	AdK (Mn <sup>2+</sup> /Mg <sup>2+</sup> )	PEP		3, no reactn
UTP	AdK	3-PGA	12 (92)	5
	NMPK	3-PGA	10 (58)	10; incomplete
	NMPK	PEP	6 (92)	1
	AdK (Mn <sup>2+</sup> )	PEP	0.92 <sup>c</sup>	3
	AdK	PEP	1.16 <sup>c</sup>	3
	AdK (Mn <sup>2+</sup> /Mg <sup>2+</sup> )	PEP	0.92 <sup>c</sup>	3
ATP	AdK	3-PGA	9 (91)	1

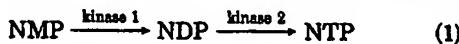
<sup>a</sup>Magnesium(II) was present in all reactions unless noted otherwise. AdK = adenylate kinase (EC 2.7.4.3); GK = guanylate kinase (EC 2.7.4.8); NMPK = nucleosidemonophosphate kinase (EC 2.7.4.4). <sup>b</sup>3-PGA = D-(–)-3-phosphoglyceric acid; PEP = phosphoenolpyruvate.

<sup>c</sup>Product was not assayed.

because it has already been synthesized enzymatically on a 50-mmol scale.<sup>6</sup>

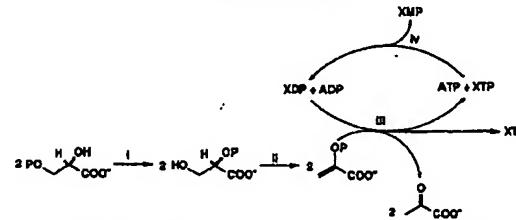
**Objective.** Our objective in this work was to develop convenient syntheses on ~10-g scale of CTP, GTP, and UTP of sufficient purity for use in enzyme-catalyzed reactions. Four strategies can be used to produce NTPs: (1) enzymatic synthesis (using cell-free enzymes), (2) chemical synthesis, (3) fermentation, and (4) isolation from natural sources. We considered the latter two methods to be too unfamiliar to be useful in classical synthetic organic chemistry laboratories and did not investigate their merits. We conclude that enzymatic methods provide the most convenient routes to CTP and GTP. Chemical deamination of CTP (produced enzymatically) is the best route to UTP.

**Methods of Enzymatic Synthesis.** Enzymatic conversion of a NMP to a NTP requires two kinases: one for NMP and one for NDP (eq 1). The synthesis of NTPs



from NDPs is straightforward. Three kinases are available that convert all four of the NDPs (ADP, CDP, GDP, and UDP) to the corresponding NTPs: pyruvate kinase<sup>7</sup> (PK, EC 2.7.1.40) uses phosphoenolpyruvate (PEP) as a phosphoryl donor, acetate kinase<sup>8</sup> (EC 2.7.2.1) uses acetyl phosphate, and nucleosidediophosphate kinase<sup>9</sup> (EC 2.7.4.6) uses ATP. We chose pyruvate kinase as kinase 2 because PEP is more stable than acetyl phosphate and pyruvate kinase is less expensive than nucleosidediophosphate kinase.<sup>5</sup>

The preparation of NDPs from NMPs is more difficult than the preparation of NTPs from NDPs. No one, stable, inexpensive enzyme is known that converts all of the NMPs to NDPs. We examined three commercially available kinases: adenylate kinase<sup>6</sup> (AdK, EC 2.7.4.3), guanylate kinase<sup>7</sup> (GK, EC 2.7.4.8), and nucleosidemonophosphate kinase<sup>10</sup> (NMPK, EC 2.7.4.4). In vivo, adenylate kinase phosphorylates AMP and guanylate kinase<sup>7</sup> phosphorylates GMP. Adenylate kinase also phosphorylates CMP at synthetically useful rates.<sup>9,10</sup> Two other specific

Scheme I.<sup>a</sup> Enzymatic Synthesis of Nucleoside Triphosphates

<sup>a</sup>(i) Phosphoglycerate mutase (EC 2.7.5.3); (ii) enolase (EC 2.4.1.11); (iii) pyruvate kinase (EC 2.7.1.40); (iv) adenylate kinase (EC 2.7.4.3, X = A, C, U), guanylate kinase (EC 2.7.4.8, X = G) or nucleosidemonophosphate kinase (EC 2.7.4.4, X = U). P = phosphate. Table I lists scales and yields.

kinases, cytidyl kinase (EC 2.7.4.14) and uridyl kinase<sup>7</sup> (EC 2.7.1.48) are not commercial products. Nucleosidemonophosphate kinase uses ATP to phosphorylate AMP, CMP, GMP, and UMP.<sup>11</sup>

A serious drawback to the use of NMPK is its instability and cost. Furthermore, preparations of NMPK are not homogeneous, and a mixture of kinases may actually be present. Because adenylate kinase is the least expensive and most stable of these three kinases, we tried to use it to phosphorylate UMP and GMP. We were able to convert UMP to UDP using adenylate kinase, but not GMP to GDP.

Many kinases use ATP as a phosphorylating agent. ATP usually is recycled from ADP by using pyruvate kinase and PEP<sup>12,13</sup> or acetate kinase and acetyl phosphate.<sup>14</sup> A recent review summarizes the relative merits of these two methods to regenerate ATP in organic synthesis.<sup>5</sup> PEP is more stable in solution than is acetyl phosphate and is thermodynamically a stronger phosphoryl donor.<sup>5</sup> Commercial PEP is, however, too expensive (\$4800/mol) to use in reactions on a preparative scale (>50 mmol of PEP is required for the larger reactions described in this paper), so it must be synthesized in a separate step. For this work, we developed a convenient method (the PGA method, Scheme I) for the enzymatic synthesis of PEP from the relatively inexpensive D-(–)-3-phosphoglyceric acid (3-PGA, \$260/mol).<sup>15</sup>

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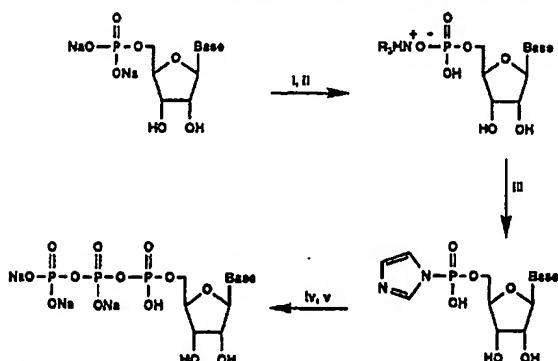
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**Scheme II.<sup>a</sup> Chemical Synthesis of Nucleoside Triphosphates Using the Carbonyldiimidazole Method**



<sup>a</sup> (i) Dowex H<sup>+</sup>; (ii) R<sub>3</sub>N (R = CH<sub>3</sub>(CH<sub>2</sub>)<sub>2</sub>, or CH<sub>3</sub>(CH<sub>2</sub>)<sub>3</sub>), MeOH/EtOH; (iii) carbonyldiimidazole, CH<sub>3</sub>CN, 25 °C, 1 day; (iv) [CH<sub>3</sub>(CH<sub>2</sub>)<sub>3</sub>]<sub>2</sub>N<sup>+</sup>H<sub>2</sub>P<sub>2</sub>O<sub>7</sub><sup>-</sup>, CH<sub>3</sub>CN, 1 day; (v) NaClO<sub>4</sub>, (CH<sub>3</sub>)<sub>2</sub>CO, base = adenosine, cytidine, guanosine, or uridine. Table II lists scales and yields.

**Methods of Chemical Synthesis.** A large repertoire of chemical methods for the preparation of NTPs from NMPs is available.<sup>18-20</sup> We chose to activate the NMPs as nucleoside imidazolates<sup>21-24</sup> (using the carbonyldiimidazole method, Scheme II) because the reaction of NMPs with 1,1'-carbonyldiimidazole occurs under relatively mild conditions compared with other methods and does not require a purification step. Other methods of chemical synthesis may be equally satisfactory.

### Results and Discussion

**Enzymatic Synthesis.** Table I summarizes the scales and yields for enzymatic syntheses.

**CTP. Adenylate Kinase/Pyruvate Kinase Method.** The AdK/PK method consistently produced ~25 mmol of CTP starting from 10 g of CMP, 2 equiv of PEP, and a catalytic amount of ATP.<sup>9</sup> For larger scale reactions (200 mmol), we found it more convenient, albeit more expensive,<sup>25</sup> to generate PEP in situ using the PGA method than to synthesize PEP in a separate chemical step.

The consumption of HCl during the course of the reaction provided a convenient way to monitor the progress of the reaction.<sup>26</sup> Analysis by <sup>31</sup>P NMR and <sup>1</sup>H NMR spectroscopy also allowed the conversion of CMP to CTP

**Table II. Scale and Yields for Chemical Synthesis of Nucleoside Triphosphates Using the Carbonyldiimidazole Method**

NTP	amount, g (yield, %) <sup>a</sup>	NTP	amount, g (yield, %) <sup>a</sup>
ATP	4.1 (80) <sup>b</sup>	CTP	0.59 (73) <sup>c</sup>
GTP	4.2 (78) <sup>b</sup>	UTP	0.62 (76) <sup>c</sup>

<sup>a</sup> After ion-exchange chromatography. Purity >95% according to analysis by <sup>1</sup>H and <sup>31</sup>P NMR spectroscopy. <sup>b</sup> Tri-n-butylammonium salt. <sup>c</sup> Tri-n-octylammonium salt.

to be followed (Figure 1, C and D). Simple precipitation with ethanol (1:1, v/v) provides CTP (and the other NTPs) of sufficient purity for use in enzyme-catalyzed synthesis. Analysis by <sup>31</sup>P and <sup>1</sup>H NMR spectroscopy indicated that ~1% each of ATP, dipyruvate, 3-PGA, and ethanol were also present (Figure 1). If pure material were required, many purification methods based on ion-exchange chromatography exist (for examples, see the part of the Experimental Section describing chemical preparations of nucleoside triphosphates). Preparation of ATP in a similar manner using PEP generated in the reaction mixture from 3-PGA was also successful.

A catalytic amount of ATP or CTP was needed to initiate the reaction catalyzed by adenylate kinase (Scheme I). The subsequent reaction catalyzed by pyruvate kinase forms 1 equiv of CTP and regenerates either triphosphate. In the synthesis of CTP, we used a catalytic amount of ATP rather than CTP because ATP is less expensive than CTP and because the value of  $k_{cat}/K_m$  with pyruvate kinase for ADP (~1.4 × 10<sup>6</sup>, pH 7.5) is greater than the corresponding value for CDP (~1.2 × 10<sup>4</sup>, pH 7.5).<sup>27</sup> In practice, the reaction is indeed qualitatively faster when ATP rather than CTP initiates the reaction.

Two additional operational details are worth noting. First, we did not use the MEEC<sup>28</sup> technique because, on a 10-g scale, transport of the nucleoside phosphates across the regenerated cellulose acetate membrane proceeded at an inconveniently slow rate. Second, in early experiments, we added bovine serum albumin (BSA, 1–10 mg/mL) to stabilize the soluble enzymes,<sup>29</sup> but, based on qualitative observations, the presence of BSA is not necessary.

**UTP. Nucleoside Monophosphate Kinase/Pyruvate Kinase Method.** We made UTP using the NMPK/PK method, but NMPK deactivated rapidly. Others have noted the instability of NMPK and have increased its stability by immobilizing the enzyme.<sup>30,31</sup> The AdK/PK method yielded UTP in the presence of Mg<sup>2+</sup> in 1-g scale experiments, but the rate of reaction was too slow to be useful on a 10-g scale unless large amounts of enzyme were used. We tried replacing Mg<sup>2+</sup> with Mn<sup>2+</sup> and mixtures of Mg<sup>2+</sup> and Mn<sup>2+</sup> but we did not observe a useful change in the rate of the reaction.

**GTP. Guanylate Kinase/Pyruvate Kinase Method.** We made GTP using either PEP or 3-PGA as the ultimate phosphoryl donor using guanylate kinase. We used less Mg<sup>2+</sup> (~0.5 equiv) than in other experiments because GMP precipitated when more Mg<sup>2+</sup> was added. The major impurity in the GTP produced enzymatically is GDP. GTP is unstable,<sup>32</sup> and we did observe decomposition of

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 (25) Estimated cost (based on research-scale quantities of reagents from US Biochemical or Aldrich) for the chemical synthesis<sup>18</sup> of 1 mol of PEP from 2 mol of pyruvate, 2 mol of bromine, and 1.9 mol of trimethyl phosphite, \$44; from 1 mol of 3-PGA (\$250) using 5000 U each of phosphoglycerate mutase, enolase, and pyruvate kinase, \$295. In the synthesis of CTP described in this paper, the amounts of enzymes were not optimized to minimize the cost; adenylate kinase is the limiting reagent so one would not need twice as much of the other enzymes to make 1 mol of PEP.

(26) The net change in charge of the species present explains the need for HCl:  $\text{ROPO}_3^{2-} + 2\text{CH}_3-\text{C}(\text{OPO}_3^{2-})\text{CO}_2^- + 2\text{H}^+ \rightarrow \text{ROPO}_3^{2-}\text{OPO}_3^{2-} + 2\text{CH}_3\text{C}(\text{O})\text{CO}_2^-$ . This expression is only approximate because of the different values of  $pK_a$  of the reactants and products and because the reaction solutions usually are buffered.

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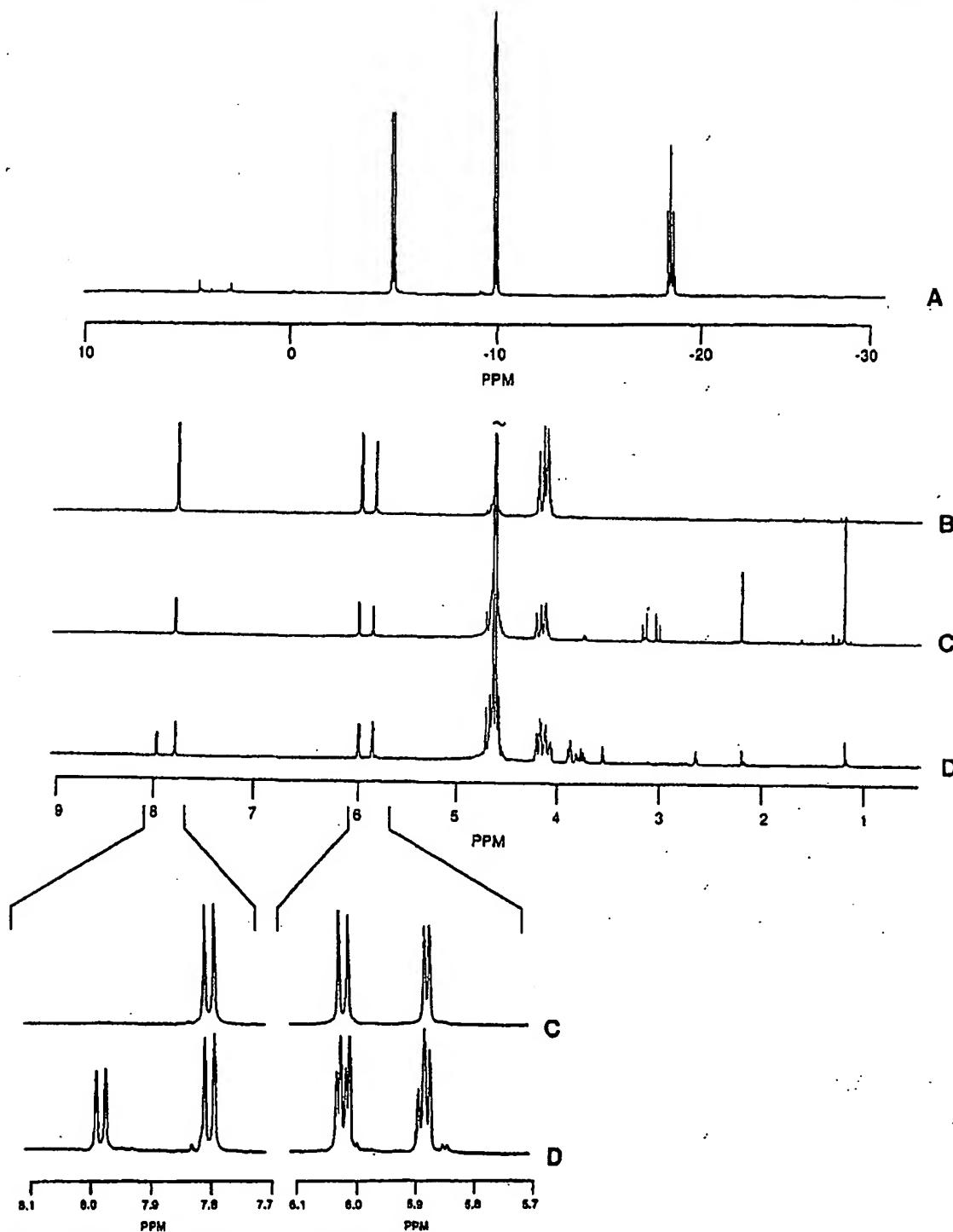


Figure 1. Reaction progress as determined by NMR spectroscopy for the synthesis of 0.2 mol of CTP from CMP and 3-PGA according to Scheme I. The solvent was  $D_2O$ ; the large peak at 4.67 ppm in the  $^1H$  NMR spectra (600 MHz) was due to DOH. (A) Decoupled  $^{31}P$  NMR spectrum of product CTP after precipitation with  $EtOH/H_2O$  (1:1, v/v). (B)  $^1H$  NMR spectrum of product CTP after precipitation with  $EtOH/H_2O$  (1:1, v/v). Most of the pyruvate, dipyruvate, and triethanolamine buffer present in the reaction mixture were removed. (C)  $^1H$  NMR spectrum (and expansion) of the reaction mixture before precipitation of CTP with  $EtOH$ . Most of the CMP and 3-PGA originally present was converted to CTP and pyruvate (a, 2.2 ppm). Some dipyruvate (s, 1.2 ppm and 2 d, ~3 ppm) also formed. (D)  $^1H$  NMR spectrum (and expansion) of the reaction mixture after 18 h at 58% conversion of CMP to CTP.

GTP to GDP (according to analysis by  $^{31}P$  NMR) during workup. Further purification of GTP before use in enzyme-catalyzed synthesis is not necessary: we used GTP

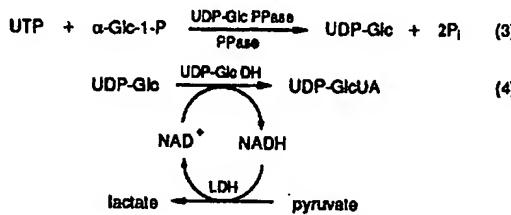
prepared from GMP to synthesize GDP-mannose (GDP-Man) in a reaction catalyzed by GDP-mannose pyrophosphorylase (GDP-Man PPase, EC 2.7.7.13) isolated



from brewer's yeast (eq 2).<sup>33</sup>

An effort to replace guanylate kinase with the less expensive adenylate kinase was not successful. We observed no production of GTP from GMP and ATP using the AdK/PK/PEP system in the presence of either  $\text{Mg}^{2+}$  or  $\text{Mn}^{2+}$ .

**Chemical Synthesis. Carbonyldiimidazole Method.** Following published procedures (Scheme II),<sup>21-24</sup> we prepared ATP, CTP, UTP, and GTP from the corresponding NMPs as tri-*n*-alkylammonium salts in quantities of 1–5 g ( $\sim$ 1–10 mmol,  $\sim$ 75% yield) after ion-exchange chromatography<sup>34</sup> on DEAE-cellulose (Table II). Chromatography may not be necessary in certain applications: for example, unpurified UTP (containing pyrophosphate salts as the major contaminant) was used in the synthesis of UDP-glucose (UDP-Glc) in the presence of  $\alpha$ -D-glucose 1-phosphate (Glc-1-P), uridine-5'-diphosphoglucose pyrophosphorylase (UDP-Glc PPase, EC 2.7.7.9), and inorganic pyrophosphatase (PPase, EC 3.6.1.1) (eq 3).<sup>35</sup> UDP-Glc dehydrogenase (UDP-Glc DH, EC 1.1.1.22) catalyzed the  $\text{NAD}^+$ -dependent oxidation of UDP-Glc to UDP-glucuronic acid (UDP-GlcUA);<sup>36</sup> a coupled reaction recycled  $\text{NAD}^+$  using pyruvate and L-lactate dehydrogenase (LDH, EC 1.1.1.27) (eq 4).<sup>5</sup>



Yields of the NTPs were approximately the same when either the tri-*n*-butylammonium salt or the tri-*n*-octylammonium salt of the NMPs were used. We used the tri-*n*-butylammonium salts for larger scale reactions because they are less expensive to prepare than the tri-*n*-octylammonium salts.

Several modifications of the published procedures improved the preparation of NTPs using the carbonyldiimidazole method. The use of acetonitrile rather than dimethylformamide as the solvent simplified the workup, because acetonitrile is the more easily removed by evaporation. We found that only 4 molar equiv of 1,1'-carbonyldiimidazole was required per equiv of NMP, rather than the reported five;<sup>24</sup> using fewer than 4 equiv resulted in lower yields. Expensive, crystallized tri-*n*-butylammonium pyrophosphate is not required; we prepared a 1 M solution of this reagent without using ion-exchange chromatography simply by dissolving anhydrous pyrophosphoric acid and tri-*n*-butylamine in acetonitrile.

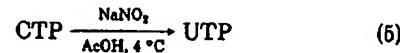
**UTP. Deamination of CTP.** Chemical deamination of CTP to UTP at 4 °C using sodium nitrite in aqueous

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acetic acid converted CTP to UTP (eq 5).<sup>37,38</sup> We noted some decomposition of UTP to UDP and UMP according to analysis by thin-layer chromatography when the deamination was performed at room temperature.

This method is more convenient than the enzymatic synthesis of UTP from UMP. UTP obtained by this deamination route was used to produce UDP-glucose using UDP-Glc PPase (eq 3). The successful synthesis of UDP-Glc using UTP prepared by this procedure establishes that any  $\text{NO}_2^-$  carried through the purification does not deactivate UDP-Glc PPase (or, we presume, other enzymes).

Because NMPK is expensive and CTP easily obtained, we also examined the enzymatic deamination of CTP to UTP.<sup>39</sup> In model systems, deamination of CMP to UMP with adenosine deaminase (EC 3.5.4.4) or with 5'-adenylic acid deaminase (EC 3.5.4.6) was not successful. We did not try to deaminate CTP using these enzymes.

**Techniques for Monitoring Reactions.** Several techniques are useful for monitoring the synthesis of NTPs. Thin-layer chromatography on PEI-cellulose is a particularly convenient analytical method.<sup>31,32</sup> The characteristic chemical shifts and coupling patterns in the phosphorus and proton NMR spectra also allow quantitative analysis of the course of the reactions. Several methods using HPLC have also been described.<sup>3,31,40</sup>

**Purification.** Many reports describe the purification of NTPs by ion-exchange chromatography.<sup>34,41</sup> We did not require analytically pure material and simply precipitated the NTPs by adding ethanol (1:1, v/v) following the enzyme-catalyzed reactions. Pyruvate and contaminating inorganic and buffer salts do not precipitate to a significant extent under these conditions.<sup>42</sup> The NTPs obtained in this way can be used in enzyme-catalyzed reactions without further purification. If pure material is desired, this initial precipitation step simplifies purification by ion-exchange chromatography.

**Manipulation of Enzymes.** The intent of this study was to develop methods to synthesize the NTPs that could be performed conveniently in organic chemistry laboratories. The enzymes were treated as off-the-shelf reagents and were neither assayed nor immobilized (although all of the enzymes used have been immobilized in other work).<sup>30,31,43</sup> We note that soluble enzymes can be recovered by ultrafiltration.<sup>44</sup> We did add an antioxidant (2-mercaptoethanol or dithiothreitol) and performed the reactions in an atmosphere of nitrogen because most of the enzymes used have air-sensitive thiol groups. We preferred to use enzymes obtained as lyophilized powders to avoid

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(42) According to analysis by  $^1\text{H}$  NMR spectroscopy, most of the buffer salts do not precipitate. Precipitates do not form in solutions of ethanol/water (1:1) at 4 °C containing 1 M  $\text{KCl}$  and 1 M  $\text{MgCl}_2$ .

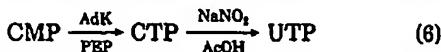
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the precipitation of magnesium ammonium phosphate salts that often occurs when suspensions in ammonium sulfate are used.

### Conclusion

Methods primarily based on enzymatic synthesis rather than on chemical synthesis are most convenient for the synthesis of CTP and GTP (eq 6 and eq 7).<sup>45</sup> UTP is best



synthesized from CMP by using a two-step procedure involving both enzymatic and nonenzymatic steps (eq 6). The most convenient preparation of PEP for these reactions is that based on in situ conversion of 3-PGA. Simple precipitation of the NTPs with ethanol yields material of sufficient purity for use in enzyme-catalyzed synthesis. A summary of the best method to make each NTP is thus: CTP, adenylate kinase/PGA method; GTP, guanylate kinase/PGA method; UTP, deamination of CTP with NaNO<sub>2</sub>/AcOH.

### Experimental Section

**Materials and Methods.** Adenosine deaminase (from calf intestinal mucosa, EC 3.5.4.4), adenylate kinase (from chicken muscle, EC 2.7.4.3), 5'-adenylic acid deaminase (from *Aspergillus* sp., EC 3.5.4.6), enolase (from bakers' yeast, EC 4.2.1.11), guanylate kinase (from bovine brain, EC 2.7.4.8), inorganic pyrophosphatase (from bakers' yeast, EC 3.6.1.1), nucleosidemonophosphate kinase (from bovine liver EC 2.7.4.4), pyruvate kinase (from rabbit muscle, EC 2.7.1.40), and uridine 5'-diphosphoglucose pyrophosphorylase (from bakers' yeast, EC 2.7.7.9) were lyophilized powders from Sigma. Alkaline phosphatase (from *Escherichia coli*, EC 3.1.3.1), phosphoglycerate mutase (from rabbit muscle, EC 2.7.5.3), and L-lactate dehydrogenase (from rabbit muscle, EC 1.1.1.27) were crystalline suspensions in solutions of ammonium sulfate from Sigma. GDP-mannose pyrophosphorylase (EC 2.7.7.13) was isolated from brewers' yeast (US Biochemical)<sup>46</sup> and UDP-glucuronic pyrophosphorylase (EC 1.1.1.22)<sup>47</sup> was isolated from calf liver acetone powder (Sigma). Commercial enzymes were not assayed; the activities stated by the manufacturer are reported here (1 unit (U) converts 1 μmol of substrate to products per minute under assay conditions). Ion-exchange resin (Dowex 50W-X8, H<sup>+</sup> form, 20–50 mesh, unless noted otherwise) was from Bio-Rad. Chemicals and solvents were reagent grade and were used without further purification, unless noted. Water was distilled from glass in a Corning AG-1b still. CMP (free acid) and GMP (sodium salt) were each obtained in 1-kg quantity from Miwon Foods Co., Ltd., Seoul, Korea. The sodium salt of AMP was obtained in 1-kg quantity from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. D-(+)-3-Phosphoglyceric acid (3-PGA) was purchased as the barium salt from either US Biochemical Co., ICN Biochemicals, or Sigma. The potassium salt of PEP was synthesized from pyruvic acid as described.<sup>13</sup> The <sup>1</sup>H and <sup>31</sup>P NMR spectra of the nucleoside triphosphates and nucleoside diphosphate sugars (UDP-Glc, UDP-GlcUA, GDP-Man) were in accord with those of commercial samples; the products coeluted with authentic compounds when analyzed by thin-layer chromatography. The yields of nucleoside triphosphates were determined by enzymatic assay.<sup>48</sup> Reactions were conducted at room

temperature (~22–24 °C) unless noted otherwise. Addition of solutions of HCl (contained in a buret) by a peristaltic pump driven by a pH controller maintained the pH of reaction mixtures in the ranges stated. Polyethylenimine-cellulose plates for thin-layer chromatography were from Aldrich.

**Cytidine 5'-Triphosphate (AdK/PK/PGA Method).** A suspension of 173 g of 3-PGA (barium salt, dihydrate, ~95%, 461 mmol) in 500 mL of water was vigorously stirred with ~600 mL of ion-exchange resin (H<sup>+</sup> form) for 30 min. The resin was removed by filtration and washed four times with 100-mL portions of water. The combined, clear, pale-yellow filtrates were neutralized with solid KOH and used directly in the next step.

CMP (free acid, 71 g, 220 mmol), ATP-Na<sub>2</sub>3H<sub>2</sub>O (1.33 g, 2.20 mmol), MgCl<sub>2</sub>·6H<sub>2</sub>O (61 g, 250 mmol), and triethanolamine (1.9 g, 10 mmol) were added to the solution of 3-PGA and the pH was adjusted to pH 7.6 by addition of 5 M KOH. The solution (total volume of 1 L) was deaerated for 30 min with nitrogen; 2-mercaptoethanol (0.25 mL, 3 mmol) was added. Adenylate kinase (10000 U), pyruvate kinase (5000 U), enolase (4000 U), and phosphoglycerate mutase (5000 U) were then added and the solution was stirred under a positive pressure of nitrogen. Addition of 3 M HCl maintained the pH at 7.5–7.8 during the course of the reaction. After 48 h, consumption of HCl had ceased but analysis by <sup>1</sup>H NMR spectroscopy indicated that the reaction was not complete. An additional 32 g of 3-PGA (barium salt, 84 mmol) was stirred with 120 mL of ion-exchange resin (H<sup>+</sup> form), filtered, and washed, and the pH of the solution was adjusted to pH 7.6 as described above. This solution of 3-PGA was deaerated and added to the reaction mixture.<sup>47</sup> After stirring for an additional 24 h, analysis by <sup>1</sup>H NMR spectroscopy indicated that the reaction was complete. A total of 130 mL of 3 M HCl had been consumed. The total amount of 3-PGA (barium salt) added was 205 g (544 mmol).

For isolation of CTP, ~150-mL portions of the clear, pale-yellow reaction mixture were transferred into twelve 500-mL polypropylene centrifuge tubes and 150 mL of absolute ethanol was added to each tube. A white precipitate formed immediately. The tubes were cooled in an ice bath for 15 min (final temperature of the ethanol–water solutions was 6 °C) and then centrifuged (4 °C, 10000g, 10 min). The supernatants were decanted and the combined, sticky pellets were dissolved in a total of 600 mL of water. The resulting solution was divided equally among six 250-mL centrifuge tubes and 100 mL of absolute ethanol was added to each tube. The tubes were cooled and centrifuged as above. Lyophilization of the combined pellets provided 145 g of an off-white powder containing 202 mmol of CTP (92% yield based on CMP; 90% purity for CTP-K<sub>2</sub>); the water content (7.14%) was determined by the Karl Fischer method. Thin-layer chromatography (polyethylenimine-cellulose; eluant,<sup>48</sup> 2.0 M HCOOH/2.0 M LiCl, 1:1, v/v) indicated that ATP was present in addition to CTP; neither CMP nor CDP were detected. Analysis by <sup>1</sup>H and <sup>31</sup>P NMR spectroscopy indicated that ~1% each of ATP, dipyruvate, 3-PGA, inorganic phosphate, and ethanol were present.

**Guanosine 5'-Triphosphate (GK/PK/PGA Method).** A suspension of 17 g of 3-PGA (barium salt, dihydrate, ~95%, 52 mmol) in 100 mL of water was stirred with ~100 mL of ion-exchange resin (Na<sup>+</sup> form) for 40 min. Most of the material dissolved, but a fine, silky, white suspension remained. The resin and suspension were removed by filtration and washed twice with 50-mL portions of water. The combined, clear filtrates were used directly in the next step.

CMP-Na<sub>2</sub>3H<sub>2</sub>O (10 g, 22 mmol), ATP-Na<sub>2</sub>3H<sub>2</sub>O (250 mg, 0.4 mmol), MgCl<sub>2</sub>·6H<sub>2</sub>O (1.5 g, 7.4 mmol), KCl (1.6 g, 22 mmol), and 50 mL of a 0.1 M solution of Tris buffer (pH 7.6) were added to the solution of 3-PGA. A white precipitate formed when magnesium ions were added, but it did not interfere with the reaction. The pH of the solution was adjusted to pH 7.6 by addition of 5 M NaOH, the volume was adjusted to 300 mL with water, and

(45) The estimated costs (based on research-scale quantities from US Biochemical or Aldrich) of the phosphorylating reagents required to convert 1 mol of a nucleoside monophosphate to the triphosphate according to the methods presented are comparable: based on 2.5 mol of 3-PGA, \$738 (see footnote 28); based on 4 mol of carbonyl diimidazole (\$700) and 4 mol of pyrophosphoric acid (\$84), \$764. This comparison does not account for costs of solvents and their disposal. In practice, if economic factors rather than convenience were the most important consideration, the phosphorylating reagents would be synthesized from inexpensive precursors in each case.

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(47) The two-step addition is not required by the procedure but simply reflects the fact that an insufficient amount of 3-PGA was added initially because of inadequate washing of the ion-exchange resin.

the solution was deaerated for 30 min with nitrogen. Guanylate kinase (10 U), pyruvate kinase (1000 U), enolase (500 U), and phosphoglycerate mutase (1000 U) were then added and the solution was stirred under a positive pressure of nitrogen. Addition of 1 M HCl maintained the pH at 7.5–7.7 during the course of the reaction. After 3 days, 48.6 mL of HCl had been consumed and analysis by thin-layer chromatography (polyethylenimine-cellulose; eluant, 1.0 M LiCl/0.5 M  $(\text{NH}_4)_2\text{SO}_4$ , 1:1, v/v) and  $^{31}\text{P}$  NMR spectroscopy indicated that the reaction was >95% complete.

For isolation of GTP, 350 mL of absolute ethanol was added to the solution (350 mL). The resulting precipitate was collected by centrifugation (10000g, 10 min) and was dissolved in 300 mL of water. Additional ethanol (300 mL) was added and the centrifugation step was repeated. Lyophilization of the pellet provided 12 g of a white powder containing 18 mmol of GTP (82% yield) according to enzymatic analysis (88% purity for GTP- $\text{Na}_2$ ). According to analysis by  $^{31}\text{P}$  NMR spectroscopy, some GDP formed during workup.

**Guanosine 5'-Triphosphate (GK/PK/PEP Method).** The execution of this reaction was similar to the previous one. Pyruvate kinase (1000 U) and guanylate kinase (10 U) were added to a solution of GMP- $\text{Na}_2\text{H}_2\text{O}$  (10 g, 22 mmol), PEP (5.4 g, 26 mmol), ATP- $\text{Na}_2\text{H}_2\text{O}$  (130 mg, 0.2 mmol),  $\text{MgCl}_2\text{H}_2\text{O}$  (1.0 g, 4.9 mmol), and KCl (1.64 g, 22 mmol) in 300 mL of a 0.1 M solution of Tris buffer (pH 7.5). After 1 day an additional 5.4 g of PEP was added and after 2 days more  $\text{MgCl}_2\text{H}_2\text{O}$  (1.0 g) was added. After 3 days, 25.7 mL of HCl had been consumed and analysis by  $^1\text{H}$  NMR spectroscopy indicated that the conversion of GMP to GTP was complete.

Isolation of GTP by precipitation with ethanol as described above provided 12 g of a white powder containing 19 mmol of GTP (86% yield) according to enzymatic analysis (93% purity for GTP- $\text{Na}_2$ ).

**Guanosine 5'-Triphosphate (AdK/PK/PEP/Mn<sup>2+</sup> Attempts).** Adenylate kinase (1000 U) and pyruvate kinase (1000 U) were added to a solution of 1.00 g of GMP- $\text{Na}_2\text{H}_2\text{O}$  (2.17 mmol), 100 mg of ATP- $\text{Na}_2\text{H}_2\text{O}$  (0.2 mmol), 1.1 g of PEP-K<sup>+</sup> (5.2 mmol), 250 mg of  $\text{MnCl}_2\text{H}_2\text{O}$  (1.3 mmol), and 23 mg of dithiothreitol in 50 mL of 0.1 M solution of Tris buffer (pH 7.7). Analysis by thin-layer chromatography (polyethylenimine-cellulose; eluant, 2.0 M HCOOH/2.0 M LiCl, 1:1, v/v) indicated no conversion of GMP to GTP within 3 days.

**Guanosine 5'-Diphosphate (AdK/ATP/Mn<sup>2+</sup>-Mg<sup>2+</sup> Attempts).** A solution of 100 mg of GMP- $\text{Na}_2\text{H}_2\text{O}$  (0.22 mmol), 100 mg of ATP- $\text{Na}_2\text{H}_2\text{O}$  (0.17 mmol), 50 mg of  $\text{MnCl}_2\text{H}_2\text{O}$  (0.25 mmol), 50 mg of  $\text{MgCl}_2\text{H}_2\text{O}$  (0.25 mmol), and 1 mg of dithiothreitol in 15 mL of a 0.1 M solution of Tris buffer (adjusted to pH 8) was deaerated with nitrogen and adenylate kinase (100 U) was added. Analysis by thin-layer chromatography as in the preceding experiment indicated no formation of GDP within 3 days.

**Uridine 5'-Triphosphate (NMPK/PK/PGA Method).** The reaction was performed as described for GTP using the GK/PK/PGA method. The initial solution contained UMP- $\text{Na}_2\text{H}_2\text{O}$  (10.0 g, 24 mmol), 3-PGA (19 g, 58 mmol, of the barium salt was converted to the sodium form), ATP- $\text{Na}_2\text{H}_2\text{O}$  (150 mg, 0.24 mmol),  $\text{MgCl}_2\text{H}_2\text{O}$  (5.1 g, 25 mmol), KCl (1.9 g, 25 mmol), Tris-HCl (3.15 g, 20 mmol), dithiothreitol (100 mg), adenylate kinase (10000 U), pyruvate kinase (1000 U), enolase (500 U), and phosphoglycerate mutase (1000 U) in a total volume of 200 mL of water (pH 7.6). Additional nucleoside-monophosphate kinase (8 U) was added after 5 days and after 6 days additional pyruvate kinase (1000 U), enolase (600 U), and phosphoglycerate mutase (1000 U) were added. After 8 days, 29.3 mL of 1 M HCl had been consumed and the reaction was stopped even though it was not complete. Precipitation of UTP with ethanol as described above provided 10.5 g of a white powder containing 14 mmol of UTP (58% yield) according to enzymatic analysis (73% purity for UTP- $\text{Na}_2$ ).

**Uridine 5'-Triphosphate (NMPK/PK/PEP Method).** The reaction was performed as described for GTP using the GK/PK/PEP method. The reaction solution contained UMP- $\text{Na}_2\text{H}_2\text{O}$  (5.0 g, 12 mmol), PEP (6.0 g, 29 mmol), ATP- $\text{Na}_2\text{H}_2\text{O}$  (73 mg, 0.12 mmol),  $\text{MgCl}_2\text{H}_2\text{O}$  (2.5 g, 12 mmol), dithiothreitol (46 mg), nucleoside-monophosphate kinase (8 U), and pyruvate

kinase (1000 U) in a total volume of 100 mL of 0.1 M solution of Tris buffer (pH 7.6). After 24 h, 21.4 mL of 1 M HCl had been consumed and analysis by  $^{31}\text{P}$  NMR indicated that the reaction was complete. Precipitation of UTP with ethanol as described above provided 6.3 g of a white powder containing 11 mmol of UTP (92% yield) according to enzymatic analysis (>95% purity for UTP- $\text{Na}_2$ ).

**Uridine 5'-Triphosphate (AdK/PK/PGA Method).** The reaction was performed as described for GTP using the GK/PK/PGA method. The initial solution contained UMP- $\text{Na}_2\text{H}_2\text{O}$  (10.0 g, 24 mmol), 3-PGA (19 g, 58 mmol, of the barium salt was converted to the sodium form), ATP- $\text{Na}_2\text{H}_2\text{O}$  (150 mg, 0.24 mmol),  $\text{MgCl}_2\text{H}_2\text{O}$  (5.1 g, 25 mmol), KCl (1.9 g, 25 mmol), Tris-HCl (3.15 g, 20 mmol), dithiothreitol (100 mg), adenylate kinase (10000 U), pyruvate kinase (1000 U), enolase (500 U), and phosphoglycerate mutase (1000 U) in a total volume of 200 mL of water (pH 7.7). After 5 days, 36.0 mL of 1 M HCl had been consumed. Precipitation of UTP with ethanol as described above provided 12 g of a white powder containing 22 mmol of UTP (92% yield, >95% purity for UTP- $\text{Na}_2$ ).

**Adenosine 5'-Triphosphate (AdK/PK/PGA Method).** The reaction was performed as described for GTP using the GK/PK/PGA method. The initial solution contained AMP- $\text{Na}_2\text{H}_2\text{O}$  (8.2 g, 20 mmol), 3-PGA (16 g, 44 mmol, of the barium salt was converted to the sodium form), ATP- $\text{Na}_2\text{H}_2\text{O}$  (100 mg, 0.17 mmol),  $\text{MgCl}_2\text{H}_2\text{O}$  (4.0 g, 20 mmol), KCl (1.5 g, 20 mmol), Tris-HCl (400 mg), dithiothreitol (100 mg), adenylate kinase (1000 U), pyruvate kinase (1000 U), enolase (500 U), and phosphoglycerate mutase (1000 U) in a total volume of 100 mL of water (pH 7.7). After 1 day, 29.6 mL of 1 M HCl had been consumed. Precipitation of ATP with ethanol as described above provided 9 g of a white powder containing 16 mmol of ATP (80% yield) according to enzymatic analysis (>95% purity for ATP- $\text{Na}_2$ ).

**Chemical Syntheses; General Procedures. Free Acids of Nucleoside Monophosphates.** Each nucleoside monophosphate disodium salt (1.0 mmol) was dissolved in 25 mL of water and stirred with 5 mL of ion-exchange resin (Dowex 50W-X8,  $\text{H}^+$  form, 50–100 mesh) for 1 h. The solution was decanted and the resin was washed 5 times with 50-mL portions of water. Rotary evaporation of the combined aqueous solutions at reduced pressure provided the free acids as amorphous powders.

**Tri-*n*-butylammonium Salts of Nucleoside Monophosphates.** The free acid of a nucleoside monophosphate (1.0 mmol) was suspended in a mixture of 10 mL of MeOH and 10 mL of EtOH, tri-*n*-butylamine (185 mg, 1.0 mmol) was added, and the reaction mixture was refluxed until the solid dissolved (~1 h). The solution was cooled and evaporated. The residue was dried by repeated addition and evaporation of 10 mL of dioxane. The salt was obtained in quantitative yield after further drying at ~0.1 Torr over  $\text{CaSO}_4$ .

**Standard Solution of Tri-*n*-butylammonium Pyrophosphate.** A suspension of anhydrous pyrophosphoric acid (17.8 g, 0.10 mol) in 60 mL of acetonitrile in a 100-mL volumetric flask was cooled in an ice bath and tri-*n*-butylamine (18.5 g, 0.10 mmol) was added. Once the solid dissolved (~1 h), the solution was allowed to warm to room temperature. Addition of acetonitrile to a final volume of 100 mL provided a 1.0 M standard solution of tri-*n*-butylammonium pyrophosphate.

**Preparation of Nucleoside Triphosphates.** The following reaction was performed under an atmosphere of argon. The nucleoside monophosphate tri-*n*-butylammonium salt (1 mmol) and carbonyldiimidazole (4 mmol) were placed in a flame-dried flask sealed with a silicone septum. Acetonitrile (20 mL) was added and the reaction mixture was stirred for 1 day. MeOH (3 mmol) was then added. After 30 min, an aliquot of the standard pyrophosphate solution (4 mL, 4 mmol) was added. After 1 day, the solvent was removed by rotary evaporation at reduced pressure and the residue treated with 20 mL of MeOH. The resulting precipitate was removed by filtration and washed with ~10 mL of MeOH. The combined solutions were concentrated to ~25 mL and a saturated solution of  $\text{NaClO}_4$  in acetone was added (~20 mL) followed by diethyl ether (5 mL). The resulting precipitate contains the sodium salts of the nucleoside triphosphate and pyrophosphoric acid. In the case of UTP, this mixture was used directly in the synthesis of UDP-Glc. The nucleoside triphosphates were purified by anion-exchange chromatography

(DEAE cellulose; eluant, a gradient of triethylammonium bicarbonate, 0.1 to 0.4 M in 3 L, pH 7.5). Triethylammonium bicarbonate was removed by repeated addition and evaporation of ethanol. The triethylammonium salts were dissolved in a small amount of methanol (~20 mL) and 10 mL of a saturated solution of  $\text{NaClO}_4$  in acetone was added to obtain the sodium salt. The precipitated sodium salt was collected by filtration and dried in vacuo over  $\text{CaSO}_4$ . Table II records the yields obtained for ATP, CTP, UTP, and GTP.

**Uridine 5'-Triphosphate (Chemical Deamination Method).** Solutions in this experiments were made by using cold (4 °C) water. The reaction was performed at 4 °C. To a solution of CTP (10 g, 14 mmol, prepared using adenylate kinase as described above) in 50 mL of water, was added a solution of  $\text{NaNO}_2$  (15 g, 21.7 mmol) in 40 mL of cold water. A solution of 1:1 (v/v) acetic acid/water (30 mL) was added and the solution was stirred. After 48 h, 150 mL of ethanol was added and the resulting precipitate was collected by centrifugation (5000g, 10 min) and redissolved in 100 mL of water. The precipitation step with ethanol (100 mL) was repeated, the precipitate obtained after centrifugation was dissolved in 25 mL of water, and the pH of the solution was adjusted to pH 7.5 with 5 M NaOH. Lyophilization of this solution provided 7.8 g of a white powder containing 14 mmol of UTP (>95% yield) according to enzymatic analysis (>95% purity for UTP- $\text{Na}_3$ ).

**UDP-glucose.** UTP synthesized by chemical deamination of CTP and by chemical synthesis (before purification by ion-exchange chromatography) was transformed into UDP-glucose according to the following general procedure. A solution of UTP (100 mg) and  $\alpha$ -D-glucose 1-phosphate (100 mg, disodium salt, excess) in 5 mL of a 0.1 M solution of Tris buffer (pH 7.8, 4 mmol of  $\text{MgCl}_2$ ) was placed in a 10-mm NMR tube containing 1 mL of  $\text{D}_2\text{O}$  and the solution was deaerated with argon for at least 10 min. A  $^{31}\text{P}$  NMR spectrum was recorded, and 10–50 U of uridine-5'-diphosphoglucose pyrophosphorylase and 10–50 U of inorganic pyrophosphatase were added. Analysis by  $^{31}\text{P}$  NMR spectroscopy indicated the formation of UDP-Glc (confirmed by comparison with an authentic sample) and inorganic phosphate and the disappearance of UTP and some of the Glc-1-P.

**UDP-glucuronic Acid.** UDP-glucose dehydrogenase was isolated from calf liver acetone powder according to the first five steps of the procedure described by Strominger et al.<sup>38,43</sup> A mixture of UDP-Glc DH (10 U), UDP-Glc (60 mg, 0.1 mmol),  $\text{NAD}^+$  (15 mg, 0.02 mmol), pyruvate (15 mg, 0.15 mmol), and L-LDH (100 U) was dissolved in 4 mL of a 0.05 M solution of Tris buffer (pH 7.5). The solution was adjusted to pH 8, flushed with argon, and stirred at room temperature. Addition of 0.5 M NaOH maintained the pH at 7.5 to 8.2 during the course of the reaction. The progress of the reaction was monitored by thin-layer chromatography (polyethylenimine-cellulose; eluant,<sup>34</sup> 1 M AcOH/4 M LiCl, 8:2, v/v). After 1 day, most of the UDP-Glc had been converted to UDP-GlcUA. The reaction mixture was applied to a column of ion-exchange resin (AG 1-X2, bicarbonate form, 200–400 mesh, 1.5 cm × 4 cm). The column was washed with 25 mL of water and 25 mL of 0.25 M  $\text{NH}_4\text{HCO}_3$  and then eluted with

a 0.5 to 1 M gradient of  $\text{NH}_4\text{HCO}_3$ . The fractions containing UDP-GlcUA were pooled and concentrated to give a white powder. Excess ammonium bicarbonate was removed by dissolving the powder in 5 mL of water and adding ion-exchange resin (Dowex 50W-X8, 20–50 mesh,  $\text{H}^+$  form) to the stirred solution until the pH was 7. The solution was filtered and lyophilized to give 41 mg of UDP-GlcUA (68% yield).

**GDP-mannose.** An extract of GDP-mannose pyrophosphorylase was prepared from 40 g of brewers' yeast according to the method of Munch-Peterson<sup>35</sup> and dissolved in 5 mL of a 0.05 M solution of Tris buffer (pH 7.5). To this solution were added 0.05 mL of a 1 M solution of sodium fluoride, 0.45 mL of a 0.1 M solution of EDTA, 92 mg of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (0.45 mmol), followed by a solution of 51 mg of  $\alpha$ -mannose 1-phosphate (di-cyclohexylammonium salt, 0.10 mmol),<sup>36</sup> and 140 mg of GTP (0.24 mmol) in 1 mL of a 0.05 M solution of Tris buffer (pH 7.5). The reaction mixture was stirred at room temperature and monitored by thin-layer chromatography (polyethylenimine-cellulose; eluant,<sup>34</sup> 1 M AcOH/3 M LiCl, 9:1, v/v). After 24 h, the reaction flask was placed in a bath of boiling water for 1.5 min and the resulting precipitate was removed by centrifugation (7000g, 10 min). The supernatant was stirred with 150 U of alkaline phosphatase at 37 °C for 90 min<sup>30,31</sup> and then applied to a column of ion-exchange resin (Dowex 1X2-400, formate form, 200–400 mesh, 1 cm × 25 cm). The column was washed with 150 mL of water and 500 mL of 2 M formic acid. The eluants were discarded. Elution of the column with 4 M formic acid (450–500 mL) and lyophilization of the eluant gave a residue containing GDP-Man. The residue was dissolved in 10 mL of water and stirred for 10 min with ~1 g of ion-exchange resin (Dowex 50W-X8,  $\text{H}^+$  form). The solution was decanted and the resin was washed three times with 10-mL portions of water. The pH of the combined solutions was adjusted to pH 8.0 with a 1 M solution of NaOH. Lyophilization of the resulting solution gave 38 mg of GDP-Man (0.055 mmol, 54% yield).

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**Registry No.** 3-PGA-Ba- $2\text{H}_2\text{O}$ , 86879-11-0; CMP, 63-37-6; CMP- $\text{Bu}_3\text{N}$ , 51450-21-6; GMP, 85-32-5; GMP-2Na, 5550-12-9; GMP- $\text{Bu}_3\text{N}$ , 75252-11-8; UMP, 58-97-9; UMP-2Na, 3387-36-8; UMP- $\text{Bu}_3\text{N}$ , 87713-40-4; AMP, 61-19-8; AMP-2Na, 4578-31-8; AMP- $\text{Bu}_3\text{N}$ , 59618-80-3; ATP-2Na- $3\text{H}_2\text{O}$ , 51963-81-2; ATP-3Na, 20978-32-9; CTP-3K, 124992-46-7; CTP-3Na, 54619-78-2; GTP-3Na, 38051-31-7; UTP-3Na, 19817-92-6; PEP, 138-08-9; UDP-Glc, 133-89-1; UDP-GlcUA, 2818-64-0; GDP-Man, 3123-67-9; Glc-1-P-2Na, 56401-20-8; Man-1-P-( $\text{C}_6\text{H}_{11}\text{)_2\text{NH}$ , 72948-45-3; triethylammonium pyrophosphate, 50859-18-2.

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## Combined Enzymatic Synthesis of Nucleotide (Deoxy) Sugars from Sucrose and Nucleoside Monophosphates

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**Abstract:** The synthesis of NDP-glucose 3a-d (N= A, C, U, dU) with sucrose synthase B was combined with the enzymatic synthesis of nucleoside diphosphates 2a-d from their corresponding nucleoside monophosphates 1a-d by different kinases A. Further combination with recombinant dTDP-glucose 4,6-dehydratase D enabled us to synthesize dUDP-6-deoxy- $\alpha$ -D-xylo-4-hexulose 5 from 1d on a preparative scale. By using the repetitive batch technique the enzymatic syntheses of nucleotide (deoxy) sugars 3a-d, 5 could be realized on a 0.1 - 0.5 g scale.

### INTRODUCTION

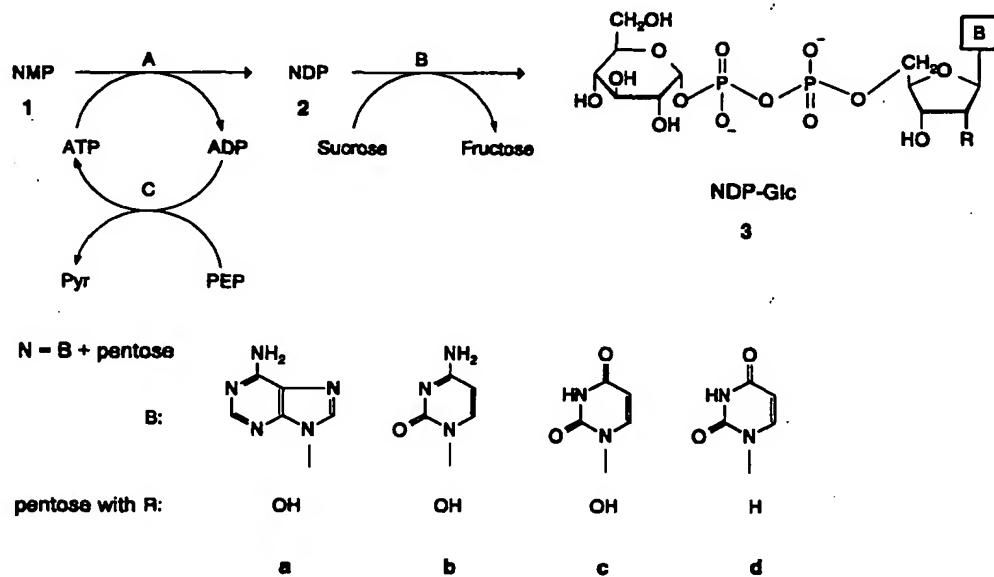
During the last decade it became evident that oligosaccharide residues of glycoconjugates are involved in important intra- and intermolecular communication events leading to an increased demand for glycoconjugates as research and therapeutic targets.<sup>1</sup> Since the chemical synthesis of oligosaccharides is quite difficult and tedious including the complex protection and deprotection chemistry of reactive groups as well as problems of stereocontrolled synthesis<sup>2</sup> glycosyltransferases and glycosidases have been applied because of their high regio- and stereoselectivity without the need for protection.<sup>3,4</sup> The utilization of glycosyltransferases requires nucleotide sugars. Apart from the chemical preparation of nucleotide sugars some large-scale, enzymatic syntheses have been developed by using highly specific pyrophosphorylases.<sup>5,6</sup> Other important nucleotide sugars such as ADP-Glc<sup>7</sup> 3a, CDP-Glc<sup>8</sup> 3b and dUDP-Glc<sup>9</sup> 3d have been synthesized enzymatically only on a small scale.

Sucrose synthase (B, SuSy, EC 2.4.1.13) from rice grains catalyzes the cleavage of sucrose 4 with uridine-5'- (UDP) 2c, 2'-deoxythymidine-5'-(dTDP), 2'-deoxyuridine-5'-(dUDP) 2d, adenosine-5'-(ADP) 2a and cytidine-5'-(CDP) diphosphate 2b to their corresponding activated glucoses 3a-d and D-fructose.<sup>10,11</sup>

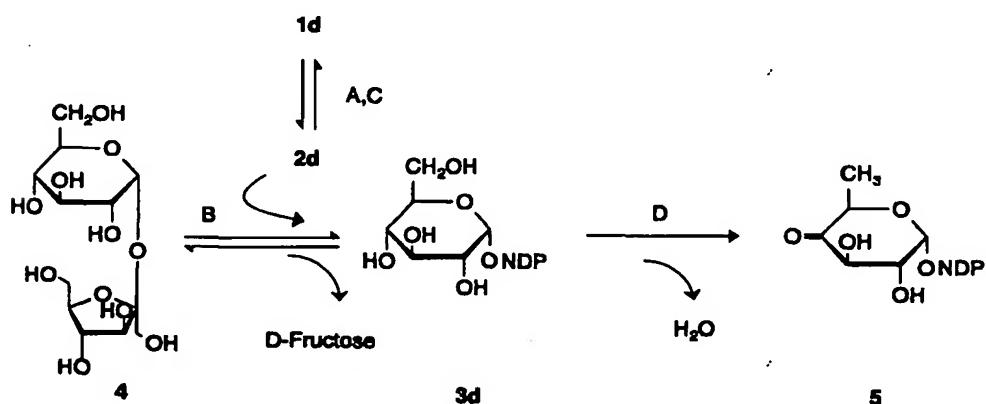
Nucleoside diphosphates (NDP) are still expensive substrates, which can be obtained from much more cheaper nucleoside monophosphates (NMP) by chemical or enzymatic synthesis. In the present paper we combined the SuSy catalyzed synthesis (B) of activated glucoses (ADP- 3a, CDP- 3b, UDP- 3c, dUDP-Glc 3d) with the enzymatic formation of NDP 2a-d from NMP 1a-d catalysed by nucleoside monophosphate kinase (A, NMPK, EC 2.7.4.4) or myokinase (A, MK, EC 2.7.4.3) including *in situ* regeneration of ATP with pyruvate kinase (C, PK, EC 2.7.1.40) (Scheme 1).

Further combination with dTDP-glucose 4,6-dehydratase (D, EC 4.2.1.46) yielded dUDP-6-deoxy- $\alpha$ -D-xylo-4-hexulose 5 in a one pot synthesis starting from 1d and 4 (Scheme 2). The synthesis of this analogue of dTDP-6-deoxy- $\alpha$ -D-xylo-4-hexulose discloses now a convenient access to the key intermediate in biosynthetic pathways of many activated D- and L-deoxysugars, e.g. dTDP-L-mycarose, dTDP-L-rhamnose or dTDP-L-dihydrostreptose.<sup>12,13</sup> dUDP-6-deoxy- $\alpha$ -D-xylo-4-hexulose 5 may be also tested as substrate or inhibitor of

glycosyltransferases involved in these pathways. Recently, we utilized **5** for the reactivation of reductively inactivated UDP-glucose 4'-epimerase (EC 5.1.3.2) during the enzymatic syntheses of *N*-acetyllactosamine and analogues thereof.<sup>14</sup>



**Scheme 1:** Synthesis of activated glucoses from nucleoside monophosphates with nucleoside monophosphate kinase or myokinase **A**, sucrose synthase **B** and pyruvate kinase **C**. NMP nucleoside monophosphate; NDP nucleoside diphosphate; NDP-Glc nucleoside diphosphate glucose; ATP adenosine-5'-triphosphate; PEP phospho(enol)pyruvate; Pyr pyruvate..



**Scheme 2:** Synthesis of dUDP-6-deoxy- $\alpha$ -D-xylo-4-hexulose **5** with nucleoside monophosphate kinase **A**, pyruvate kinase **C**, sucrose synthase **B**, dTDP-glucose 4,6-dehydratase **D**; NDP nucleoside diphosphate.

## RESULTS

In order to optimize the synthesis of activated (deoxy) sugars with sucrose synthase in combination with different kinases we tested the substrate spectrum of the kinases and different important parameters e.g. pH optimum and stability.

**Substrate specificity of kinases**

The substrate specificities of myokinases (MK) from rabbit, porcine and chicken muscle and nucleoside monophosphate kinase (NMPK) from bovine liver were tested in a photometric assay (Table 1).

**Table 1: Substrate Specificities of Different Kinases**

NMP	Relative Activity* [%]			
	MK porcine	MK chicken	MK rabbit	NMPK bovine
AMP (1a)	100	100	100	100
CMP (1b)	18	10	24	58
UMP (1c)	6	4	8	82
GMP	8	4	12	20
dUMP (1d)	4	2	8	18
dTMP	0	0	0	0

\* the relative activity is based on the activity of the enzymes with AMP

MK from rabbit muscle was chosen for the synthesis of 3a and 3b. The enzyme was unstable in diluted solution but could be stabilised by the addition of 1 mg\*ml<sup>-1</sup> BSA.<sup>15</sup> NMPK was used for the preparation of 3c,d and 5.

**Determination of pH optima for the synthesis of nucleotide sugars**

The synthesis of 3a with MK, PK and SuSy as well as the synthesis of 3d with NMPK, PK and SuSy was carried out at different pH values between 7.0 and 8.0. The pH optima for the synthesis of 3a,b with MK and 3c,d with NMPK was pH 7.5 and pH 7.2, respectively.

**Preparative Synthesis of nucleotide (deoxy) sugars**

The excellent stability of all enzymes enabled us to perform the synthesis of nucleotide (deoxy) sugars with the repetitive batch technique over several days. Figure 1 shows the course of the synthesis of 5 over four days. The enzymes were recovered from the product solution by an ultrafiltration step and directly reused by the addition of fresh substrate solution.

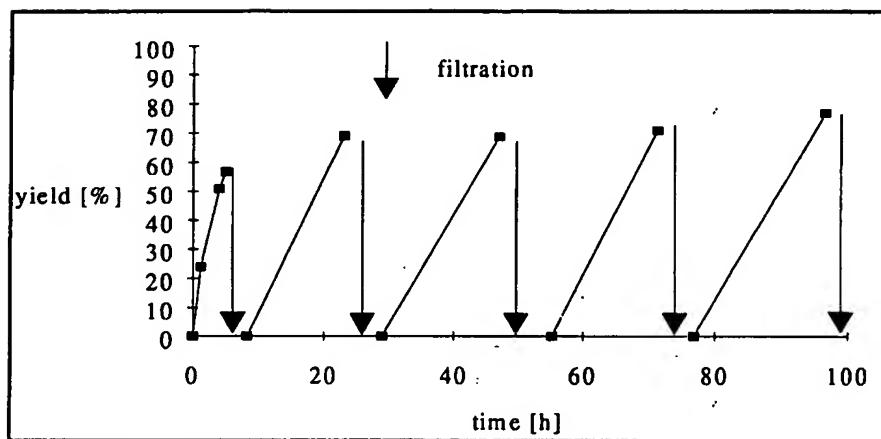


Fig. 1: Repetitive batch synthesis of dUDP-6-deoxy- $\alpha$ -D-xylo-4-hexulose 5. Arrows indicate filtration steps.

Table 2 summarizes the results of the synthesis and the isolation of nucleotide sugars which were produced by this technique. The yields for product isolation are different reflecting the different sensitivity of the nucleotide(deoxy) sugars for decomposition. However, compared to our previous method<sup>11</sup> decomposition of nucleotides by alkaline phosphatase was very efficient with a 5 - 7% loss of activated sugars. In the course of our work we found that pyruvate could be more efficiently separated by ion exchange chromatography on Dowex 1x2, 100 -200 mesh, Cl<sup>-</sup>-form, rather than by Sepharose Q FF. A small amount of 3a caused by the formation of 2a in the kinase reaction (A) and appearing during the synthesis of 3b-d can be separated by gel filtration. However, in the case of uridine-5'-diphosphate activated (deoxy) sugars adenosine-5'-triphosphate can be replaced by uridine-5'-triphosphate (UTP) in the kinase reaction (A) whereas cytidine-5'-triphosphate (CTP) and 2'-deoxyuridine-5'-triphosphate (dUTP) are not substrates of NMPK. The efficiency of the isolation protocol is especially demonstrated by the high yields of 3d and 5.

## DISCUSSION

Sucrose synthase (B) catalyses the cleavage of sucrose 4 with UDP 2c, dUDP 2d, 2'-deoxythymidine-5'-diphosphate (dTDP), ADP 2a, CDP 2b and guanosine-5'-diphosphate (GDP)<sup>10</sup>. The continuous synthesis of 2'-deoxythymidine-5'-( $\alpha$ -D-glucopyranosyl)diphosphate (dTDP-Glc) in an enzyme membrane reactor demonstrated the suitability of sucrose synthase for the synthesis of nucleotide sugars on a preparative scale<sup>11</sup>. Although nucleoside diphosphates are commercially available they are still expensive substrates compared to nucleoside monophosphates. The utilization of nucleoside monophosphate kinase or myokinase (A) enabled us to produce 3a-c in moderate and 3d in good yields starting from their corresponding nucleoside monophosphates 1a-d and 4.

GDP-Glc could not be synthesized since GDP is a poor substrate for sucrose synthase<sup>10</sup>. However, nucleoside monophosphate kinase is able to catalyse the phosphorylation of guanosine-5'-monophosphate (GMP).<sup>5</sup>

2'-Deoxythymidine-5'-monophosphate (dTMP) was not a substrate of the commercial available kinases. In this case the phosphorylation of **1d** by NMPK<sup>18</sup> is a key step in the synthesis of nucleotide deoxysugars starting from **4** and NMPs in combination with a kinase (A), sucrose synthase (B) and dTDP-glucose 4,6-dehydratase (D).

**Table 2: Nucleotide Sugar Syntheses using Repetitive Batch Technique.**

NDP-sugar	<b>3a</b>	<b>3b</b>	<b>3c</b>	<b>3d</b>	<b>5</b>
Enzymatic Synthesis					
synthesized (mg)	421	39	255	225	149
yield (%)	39	7	38	68	61
Product Isolation					
starting material (mg)	233	39	251	225	149
isolated (mg)	63	7.5	141	162	120
yield (%)	27	19	57	72	80
Overall yield (%)	<b>10</b>	<b>1</b>	<b>21</b>	<b>49</b>	<b>49</b>

In addition, the repetitive batch technique is an efficient method for the synthesis of nucleotide sugars on a preparative scale using native and expensive enzymes. Table 3 shows that the costs (chemicals and enzymes without SuSy) for the synthesis of 1 g nucleotide sugar can be efficiently reduced.

**Table 3: Estimated Costs (Chemicals and Enzymes) for Nucleotide Sugars Production without (Costs A) and with Repetitive Batch Technique (Costs B, 10 Batches)**

NDP-Glc	costs A	costs B
	[\$*g <sup>-1</sup> ]	[\$*g <sup>-1</sup> ]
<b>3a</b>	327	85
<b>3b</b>	4861	931
<b>3c</b>	2067	275
<b>3d</b>	8467	1046

## EXPERIMENTAL

### Materials

Sucrose synthase (B, SuSy) from rice grains and recombinant dTDP-Glc 4,6-dehydratase (D) (from *Salmonella enterica* group B) were purified as described elsewhere<sup>17,19</sup>. Nucleoside monophosphate kinase (NMPK, EC 2.7.4.4) from bovine liver, calf intestinal alkaline phosphatase (EC 3.1.3.1), pyruvate kinase (PK, EC 2.7.1.40) and lactate dehydrogenase (LDH, EC 1.1.1.28) from rabbit muscle were from Boehringer (Mannheim,

Germany). The nucle side m no- and diphosphates, myokinase (MK, EC 2.7.4.3) from rabbit, chicken, porcine muscle and phospho(enol)pyruvate (PEP, Na salt) were supplied by Sigma (Deisenhofen, Germany). PEP (monocyclohexylammonium, CHA-salt) and NADH were from Biomol (Hamburg, Germany). All the other chemicals were purchased from Merck (Darmstadt, Germany).

#### Analytical methods

The nucleotide sugars and the nucleotides were analyzed by ion pair HPLC<sup>20</sup>. The activity of SuSy (B) was determined for the cleavage reaction with UDP<sup>20</sup>. The dTDP-glucose 4,6-dehydratase (D) activity was assayed with 2'-deoxythymidine-5'-( $\alpha$ -D-glucopyranosyl)diphosphate (dTDP-Glc) as substrate<sup>13</sup>.

#### Photometric assay of kinases

The activity of different kinases with different nucleoside monophosphates (NMP) were determined with a photometric assay.

The assay mixture consisted of 5 mM NMP, 0.6 mM ATP, 0.8 mM PEP (Na salt), 7 U PK, 19.6 U LDH, 0.2 mM NADH, kinase (1 U MK bzw. 0.1 U NMPK) in buffer (0.1 M Tris(hydroxymethyl)aminomethane (Tris)-HCl pH 7.6, 1.2 mM MgSO<sub>4</sub>, 0.14 M KCl, 1 mg\*ml<sup>-1</sup> bovine serum albumine (BSA)). The reaction was started by the addition of appropriate diluted enzyme solution. The final volume was 1 ml. The decrease of absorption was measured at 340 nm at 37° C. One unit enzyme is the amount of enzyme which produces 1  $\mu$ mol product per minute.

#### pH-optima for the synthesis of nucleotide sugars

4 mM NMP, 6 mM PEP (Na salt), 6 mM MgCl<sub>2</sub>, 0.2 U\*ml<sup>-1</sup> SuSy, 10 U\*ml<sup>-1</sup> PK and 0.05 U\*ml<sup>-1</sup> MK from rabbit muscle (substrate: 1a) or 0.2 U ml<sup>-1</sup> NMPK (substrate: 1d) were mixed in 1 ml buffer (0.1 M Tris-HCl, 500 mM sucrose, 3 mM dithiothreitol (DTT), 1 mg\*ml<sup>-1</sup> BSA, pH: 7.0 - 8.0) and incubated for 17 h at 30° C. The reaction was stopped by separating the proteins from the substrate solution by an ultrafiltration with a centricon<sup>TM</sup>10 (Amicon, Beverly U.S.A) and analysed by HPLC.

#### Preparative Synthesis of nucleotide sugars

For all syntheses we used the repetitive batch technique<sup>16</sup>.

##### *1. 2'-Deoxyuridine-5'-( $\alpha$ -D-glucopyranosyl)diphosphate (3d)*

20 ml of 0.1 M Tris-HCl pH 7.2 with 4 mM 1d (Na salt) (I), 6 mM PEP (Na salt) (II), 0.8 mM MgCl<sub>2</sub> (III), 0.12 mM ATP (Na salt) (IV), 500 mM 4 (V), 3 mM DTT (VI) and 20 mg BSA was incubated with 40 U SuSy, 400 U PK and 20 U NMPK at 37 °C in a stirred ultrafiltration cell (Amicon, Model 8050, equipped with a membrane YM 30, cut-off 30.000 g\*mol<sup>-1</sup>). After 4 hours the solution containing the product and non-reacted substrate was separated from the enzymes by ultrafiltration. The solution was concentrated to 2 ml. 18 ml fresh substrate solution containing compounds I-VI in the same concentration was added and treated like the first batch. Eight batches were performed and 0.409 mmol (225 mg, 68%) 3d was prepared from 0.6 mmol 1d.

The solution with 0.409 mmol dUDP-Glc was incubated with alkaline phosphatase (1 U/ml product solution) for 17 hours at 30° C. The enzyme was removed by ultrafiltration. The nucleotide sugar was purified by ion-exchange using Sepharose Q FF, Cl<sup>-</sup>-form (column: 2.6 x 35.4 cm, flow rate: 4 ml\*min<sup>-1</sup>). After sample loading

the column was rinsed with dest. water before the nucleotide sugar was eluted with a LiCl-gradient (0 - 0.3 M LiCl, V = 1 l). The solution was concentrated at 25 - 30° C and 20 - 25 mbar and desalted on Sephadex G 10 (column: 2.6 x 93 cm, flow rate: 1 ml\*min<sup>-1</sup>). 251 mg product (containing 162 mg 3d and LiCl; HPLC: 98.3% 3d, 0.9% 1d, 0.8% 2'-deoxyuridine) were isolated after lyophilization. Yield: 0.294 mmol (72% based on 0.409 mmol 3d).

<sup>1</sup>H-NMR (360 MHz, D<sub>2</sub>O): δ = 8.02 (d, 1H, H-6", <sup>3</sup>J<sub>H-5",H-6" = 8 Hz); 6.41 (dd, 1H, H-1', <sup>3</sup>J<sub>H-1',H-2a,b' = 7 Hz); 6.04 (d, 1H, H-5", <sup>3</sup>J<sub>H-5",H-6" = 8 Hz); 5.68 (dd, 1H, H-1, <sup>3</sup>J<sub>H-1,H-2 = 3.4 Hz, <sup>3</sup>J<sub>H-1, P = 6.8 Hz); 4.79 (HDO); 4.69 (m, 1H, H-3'); 4.31-4.22 (m, 3H, H-4', H-5'a,b); 4.01-3.94 (m, 1H, H-5); 3.93 (m, 1H, H-6a, <sup>2</sup>J<sub>H-6a,H-6b = 12 Hz, <sup>3</sup>J<sub>H-5,H-6a = 2.2 Hz); 3.88-3.81 (m, 2H, H-6b, H-4, <sup>2</sup>J<sub>H-6a,H-6b = 12 Hz, <sup>3</sup>J<sub>H-5, H-6b = 5 Hz, <sup>3</sup>J<sub>H-3, H-4 = 9 Hz, <sup>3</sup>J<sub>H-4, H-5 = 9 Hz); 3.61 (ddd, 1H, H-2, <sup>3</sup>J<sub>H-2,H-3 = 10 Hz, <sup>3</sup>J<sub>H-1,H-2 = 3.2 Hz, <sup>4</sup>J<sub>H-2,P = 3.2 Hz); 3.53 (dd, 1H, H-3, <sup>3</sup>J<sub>H-3,H-4 = 10 Hz, <sup>3</sup>J<sub>H-2,H-3 = 10 Hz), 2.48 (m, 2H, H-2a,2b)</sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub>

<sup>13</sup>C-NMR (75 MHz, D<sub>2</sub>O): δ = 168.7 (s, C-4"); 154.1 (s, C-2"); 144.3 (s, C-6"); 104.9 (s, C-5"); 98.0 (d, C-1, <sup>2</sup>J<sub>C-1,P = 6.8 Hz), 87.9 (d, C-4', <sup>3</sup>J<sub>C-4',P = 9.0 Hz); 87.8 (s, C-1'); 75.3 (s, C-4); 75.2 (s, C-5); 74.1 (d, C-2, <sup>3</sup>J<sub>C-2,P = 8.3 Hz); 73.3 (s, C-3'); 71.7 (s, C-3); 67.9 (d, C-5', <sup>2</sup>J<sub>C-5',P = 6.0 Hz); 62.8 (s, C-6); 41.3 (s, C-2')</sub></sub></sub></sub>

ESI-MS (negative mode) m/z = 549 [M-H]<sup>-</sup>; m/z = 555 [M(Li)-H]<sup>-</sup>

## 2. Uridine-5'-( $\alpha$ -D-glucopyranosyl)diphosphate (3c)

50 ml of 0.1 M Tris-HCl pH 7.2 with 4 mM 1c (Na salt) (VII), 6 mM PEP (CHA-salt) (III), 3.2 mM MgCl<sub>2</sub> (III), 0.12 mM UTP (Na salt) (IV), 500 mM 4 (V), 3 mM DTT (VI) and 50 mg BSA was incubated with 6.3 U SuSy, 1000 U PK and 5 U NMPK at 30 °C in a stirred ultrafiltration cell as described above. After 21 hours the solution was concentrated to 5 ml. 45 ml fresh substrate solution containing compounds II, III, V, VI and VII in the same concentration was added and treated like the first batch. Six batches were performed with incubation periods between 19.5 h and 27 h. 0.451 mmol (255 mg, 38%) 3c were synthesized from 1.2 mmol 1c.

0.444 mmol 3c was taken for product isolation as described above. 402 mg product (containing 141 mg 3c, pyruvate and LiCl, HPLC: 92.6% 3c, 5.4% 1c, 2.0% Uridine) were isolated. Yield: 0.253 mmol, 57% based on 0.444 mmol 3c. For NMR analysis a part of isolated 3c was purified again by Sepharose Q FF, concentration and gelfiltration. 22.8 mg 3c (61% w/w) were isolated.

<sup>1</sup>H-NMR (300 MHz, D<sub>2</sub>O): δ = 7.96 (d, 1H, H-6", <sup>3</sup>J<sub>H-5",H-6" = 8 Hz); 5.99 (d, 1H, H-1', <sup>3</sup>J<sub>H-1',H-2' = 5 Hz); 5.99 (d, 1H, H-5", <sup>3</sup>J<sub>H-5",H-6" = 8 Hz); 5.61 (dd, 1H, H-1, <sup>3</sup>J<sub>H-1,H-2 = 3.6 Hz, <sup>3</sup>J<sub>H-1, P = 7.6 Hz); 4.79 (HDO); 4.39 (m, 2H, H-2', H-3'); 4.3 (m, 1H, H-4'); 4.24 (m, 2H, H-5'a,b); 3.94-3.87 (m, 1H, H-5); 3.87 (m, 1H, H-6a, <sup>2</sup>J<sub>H-6a,H-6b = 12.4 Hz, <sup>3</sup>J<sub>H-5,H-6a = 2.4 Hz); 3.83-3.73 (m, 2H, H-6b, H-4, <sup>2</sup>J<sub>H-6a,H-6b = 12.4 Hz, <sup>3</sup>J<sub>H-5, H-6b = 5 Hz, <sup>3</sup>J<sub>H-3, H-4 = 9 Hz, <sup>3</sup>J<sub>H-4, H-5 = 9 Hz); 3.55 (ddd, 1H, H-2, <sup>3</sup>J<sub>H-2,H-3 = 9.6 Hz, <sup>3</sup>J<sub>H-1,H-2 = 3.6 Hz, <sup>4</sup>J<sub>H-2,P = 3.2 Hz); 3.47 (dd, 1H, H-3, <sup>3</sup>J<sub>H-3,H-4 = 9 Hz, <sup>3</sup>J<sub>H-2,H-3 = 10 Hz)</sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub>

<sup>13</sup>C-NMR (75 MHz, D<sub>2</sub>O): 169.1 (s, C-4"); 154.7 (s, C-2"); 144.5 (s, C-6"); 105.5 (s, C-5"); 98.4 (d, C-1, <sup>2</sup>J<sub>C-1,P = 6.7 Hz), 91.3 (s, C-1'), 86.1 (d, C-4', <sup>3</sup>J<sub>C-4',P = 9.3 Hz); 76.6 (s, C-2'); 75.7 (s, C-4); 75.7 (s, C-5); 74.4 (d, C-2, <sup>3</sup>J<sub>C-2,P = 8.7 Hz); 72.5 (s, C-3'); 72.0 (s, C-3); 67.8 (d, C-5', <sup>2</sup>J<sub>C-5',P = 5.6 Hz); 63.2 (s, C-6)</sub></sub></sub></sub>

**3. Cytidine-5'-( $\alpha$ -D-glucopyranosyl)diphosphate (3b)**

50 ml of 0.1 M Tris-HCl pH 7.5 containing 4 mM **1b** (Na salt) (**VIII**), 6 mM PEP (CHA-salt) (**II**), 3.2 mM MgCl<sub>2</sub> (**III**), 0.12 mM ATP (Na salt) (**IV**), 500 mM **4** (**V**), 3 mM DTT (**VI**) and 50 mg BSA was incubated with 50 U SuSy, 1000 U PK and 500 U MK at 30 °C in a stirred ultrafiltration cell (Amicon, Model 8050, equipped with a membrane YM 10, cut-off 10.000 g\*mol<sup>-1</sup>). After incubation the solution was concentrated to 5 ml and the reaction was started with 45 ml of a solution containing compounds **II-VI** and **VIII**. Five batches were performed with incubation periods between 19 h and 24 h. 0.069 mmol (39 mg, 7%) **3b** were prepared from 1 mmol **1b**.

0.069 mmol **3b** were isolated as described above yielding 9.2 mg product (81% w/w **3b**, HPLC: 89% **3b**, 11% **1b**). Yield: 0.013 mmol, 19% based on 0.069 mmol **3b**.

<sup>1</sup>H-NMR (360 MHz, D<sub>2</sub>O):  $\delta$  = 8.02 (d, 1H, H-6", <sup>3</sup>J<sub>H-5",H-6" = 8 Hz); 6.2 (d, 1H, H-5", <sup>3</sup>J<sub>H-5",H-6" = 8.0 Hz); 6.07 (d, 1H, H-1', <sup>3</sup>J<sub>H-1',H-2' = 4.5 Hz); 5.66 (dd, 1H, H-1, <sup>3</sup>J<sub>H-1,H-2 = 3.6 Hz, <sup>3</sup>J<sub>H-1, P = 7.4 Hz); 4.78 (HDO); 4.44-4.23 (m, 5H, H-2', H-3', H-4', H-5'a,b); 4.0-3.92 (m, 1H, H-5); 3.92 (m, 1H, H-6a, <sup>2</sup>J<sub>H-6a,H-6b = 12.2 Hz, <sup>3</sup>J<sub>H-5,H-6a = 2.4 Hz); 3.88-3.8 (m, 2H, H-6b, H-4, <sup>2</sup>J<sub>H-6a,H-6b = 12.2 Hz, <sup>3</sup>J<sub>H-5, H-6b = 4.2 Hz); 3.61 (ddd, 1H, H-2, <sup>3</sup>J<sub>H-2,H-3 = 10.6 Hz, <sup>3</sup>J<sub>H-1,H-2 = 3.5 Hz, <sup>4</sup>J<sub>H-2,P = 3.3 Hz); 3.54 (dd, 1H, H-3, <sup>3</sup>J<sub>H-3,H-4 = 9.2 Hz, <sup>3</sup>J<sub>H-2,H-3 = 9.8 Hz)</sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub>

<sup>13</sup>C-NMR (90 MHz, D<sub>2</sub>O): 168.7 (s, C-4"); 160.3 (s, C-2"); 143.9 (s, C-6"); 99.1 (s, C-5"); 98.0 (d, C-1, <sup>2</sup>J<sub>C-1,P = 6.9 Hz); 91.7 (s, C-1'), 85.2 (d, C-4', <sup>3</sup>J<sub>C-4',P = 8.4 Hz); 76.7 (s, C-2'); 75.4 (s, C-4); 75.2 (s, C-5); 74.1 (d, C-2, <sup>3</sup>J<sub>C-2,P = 8.4 Hz); 71.8 (s, C-3'); 71.7 (s, C-3); 67.2 (d, C-5', <sup>2</sup>J<sub>C-5',P = 5.3 Hz); 62.9 (s, C-6)</sub></sub></sub></sub>

**4. Adenosine-5'-( $\alpha$ -D-glucopyranosyl)diphosphate (3a)**

100 ml of 0.1 M Tris-HCl pH 7.5 containing 4 mM **1a** (Na salt) (**IX**), 6 mM PEP (CHA-salt) (**II**), 8 mM MgCl<sub>2</sub> (**III**), 0.12 mM ATP (Na salt) (**IV**), 500 mM **4** (**V**), 3 mM DTT (**VI**) and 100 mg BSA was incubated with 100 U SuSy, 1000 U PK and 50 U MK at 37 °C. After the incubation time of 4 hours the solution was concentrated to 10 ml in a stirred ultrafiltration cell (Amicon, Model 8050, equipped with a membrane YM 10, cut-off 10.000 g\*mol<sup>-1</sup>) and the reaction was started with 190 ml of a solution containing compounds **II-VI** and **IX**. Three batches were performed with incubation periods between 4 h and 17 h resulting in the synthesis of 0.717 mmol (421 mg, 39%) **3a** from 1.84 mmol **1a**.

0.397 mmol **3a** was treated with alkaline phosphatase, ultrafiltrated and isolated by Sepharose Q FF as described above. Residual pyruvate in the preparation of **3a** was removed by ion-exchange (Dowex 1x2, Cl<sup>-</sup>-form, 100 - 200 mesh, 2.6 x 35.4 cm; flow rate: 4 ml\*min<sup>-1</sup>; gradient: 0 - 0.5 M LiCl, V = 0.5 l; **3a** was eluted with 1 M LiCl). After concentration, desalting and lyophilisation 76.7 mg product (82% **3a** w/w, HPLC: 99% **3a**) were isolated. Yield: 0.107 mmol, 27% based on 0.397 mmol **3a**.

<sup>1</sup>H-NMR (300 MHz, D<sub>2</sub>O):  $\delta$  = 8.5 (s, H-8"); 8.23 (s, H-2"); 6.1 (d, 1H, H-1', <sup>3</sup>J<sub>H-1',H-2' = 6 Hz); 5.58 (dd, 1H, H-1, <sup>3</sup>J<sub>H-1,H-2 = 3.6 Hz, <sup>3</sup>J<sub>H-1, P = 7.6 Hz); 4.78 (m, 1H, H-2'); 4.74 (HDO); 4.55 (m, 1H, H-3'); 4.41 (m, 1H, H-4'); 4.25 (m, 2H, H-5'a,b); 3.93-3.86 (m, 1H, H-5); 3.85 (m, 1H, H-6a, <sup>2</sup>J<sub>H-6a,H-6b = 12.8 Hz); 3.80-3.74 (m, 2H, H-6b, H-4, <sup>2</sup>J<sub>H-6a,H-6b = 12.8 Hz, <sup>3</sup>J<sub>H-5, H-6b = 4.8 Hz); 3.5 (ddd, 1H, H-2, <sup>3</sup>J<sub>H-2,H-3 = 9.5 Hz, <sup>3</sup>J<sub>H-1,H-2 = 3.3 Hz, <sup>4</sup>J<sub>H-2,P = 3.0 Hz); 3.43 (dd, 1H, H-3, <sup>3</sup>J<sub>H-3,H-4 = 9.0 Hz, <sup>3</sup>J<sub>H-2,H-3 = 9.5 Hz)</sub></sub></sub></sub></sub></sub></sub></sub></sub></sub></sub>

<sup>13</sup>C-NMR (75 MHz, D<sub>2</sub>O): 158.5 (s, C-6"); 155.8 (s, C-2"); 152.0 (s, C-4"); 142.7 (s, C-8"); 121.5 (s, C-5"); 98.4 (d, C-1, <sup>2</sup>J<sub>C-1,P = 6.6 Hz), 89.7 (s, C-1'), 86.7 (d, C-4', <sup>3</sup>J<sub>C-4',P = 9.3 Hz); 77.1 (s, C-2'); 75.7 (s, C-4);</sub></sub>

75.66 (s, C-5); 74.5 (d, C-2,  $^3J_{C-2,P} = 8.5$  Hz); 73.2 (s, C-3'); 72.1 (s, C-3); 68.1 (d, C-5',  $^2J_{C-5',P} = 5.6$  Hz); 63.2 (s, C-6)

*5. dUDP-6-deoxy- $\alpha$ -D-xylo-4-hexulose (5)*

25 ml of 0.1 M Tris-HCl pH 7.2 with 4 mM **1d** (Na salt) (**I**), 6 mM PEP (Na salt) (**II**), 0.8 mM MgCl<sub>2</sub> (**III**), 0.12 mM ATP (Na salt) (**IV**), 500 mM **4** (**V**), 3 mM DTT (**VI**) and 25 mg BSA was incubated with 50 U SuSy, 500 U PK, 25 U NMPK and 125 U dTDP-Glc-4,6-dehydratase at 25 °C in a stirred ultrafiltration cell (Amicon, Model 8050, equipped with a membrane YM 10, cut-off 10.000 g\*mol<sup>-1</sup>). After 5 h the solution was concentrated to 2.5 ml and 22.5 ml fresh substrate solution containing compounds **I-VI** in the same concentration was added and treated like the first batch. Five batches were performed with incubation periods between 5 h and 20 h. 0.279 mmol (149 mg, 61%) **5** were prepared from 0.456 mmol **1d**.

0.279 mmol was purified by ion-exchange (Dowex 1x2, Cl<sup>-</sup>-form, as described ADP-Glc), concentrated, desalting and lyophilized. 241 mg product (containing 120 mg **5** and LiCl, HPLC: 93%) were isolated. Yield: 0.224 mmol, 80% based on 0.279 mmol **5** (hydrate : ketoform: 4.5 : 1)

**Hydrate:**

<sup>1</sup>H-NMR (300 MHz, D<sub>2</sub>O) δ = 7.84 (d, 1 H, H-6",  $^3J_{H-5",H-6"} = 8$  Hz), 6.22 (dd, 1 H, H-1',  $^3J_{H-1',H-2'a} \approx$   $^3J_{H-1',H-2'b} = 7$  Hz), 5.86 (d, 1 H, H-5",  $^3J_{H-5",H-6"} = 8$  Hz), 5.45 (dd, 1 H, H-1,  $^3J_{H-1,P} = 6.8$  Hz,  $^3J_{H-1,H-2} = 3.4$  Hz), 4.64 (HDO), 4.5 (m, 1 H, H-3'), 4.14 - 4.06 (m, 3H, H-5'a,b, H-4'), 4.0 (q, 1 H, H-5,  $^3J_{H-5,H-6} = 6.2$  Hz), 3.69 (d, 1 H, H-3,  $^3J_{H-2,H-3} = 10$  Hz), 3.55 (ddd, 1 H, H-2,  $^3J_{H-2,H-3} = 10.2$  Hz,  $^3J_{H-1,H-2} = 3.4$  Hz,  $^4J_{H-2,P} = 3.4$  Hz), 2.36 - 2.22 (m, 2 H, H-2'a,b), 1.13 (d, 3 H, H-6,  $^3J_{H-5,H-6} = 6.2$  Hz)

<sup>13</sup>C-NMR (75 MHz, D<sub>2</sub>O) δ = 166.9 (s, C-4"), 152.2 (s, C-2"), 142.4 (s, C-6"), 103.1 (s, C-5"), 95.9 (d, C-1,  $^2J_{C-1,P} = 6.6$  Hz), 94.3 (s, C-4), 86.005 (d, C-4',  $^3J_{C-4',P} = 8.7$  Hz), 85.996 (s, C-1'), 73.7 (s, C-3), 71.4 (s, C-3'), 71.1 (d, C-2,  $^3J_{C-2,P} = 8.7$  Hz), 70.2 (s, C-5), 66.0 (d, C-5',  $^2J_{C-5',P} = 5.6$  Hz), 39.4 (s, C-2'), 11.9 (s, C-6)

**Keto form:**

<sup>1</sup>H-NMR (300 MHz, D<sub>2</sub>O) δ = 5.64 (dd, 1 H, H-1,  $^3J_{H-1,P} = 7.2$  Hz,  $^3J_{H-1,H-2} = 3.4$  Hz), 3.75 (ddd, 1 H, H-2,  $^3J_{H-2,H-3} = 10.2$  Hz,  $^3J_{H-1,H-2} = 3.4$  Hz,  $^4J_{H-2,P} = 3.4$  Hz), 1.17 (d, 3 H, H-6,  $^3J_{H-5,H-6} = 6.4$  Hz)

<sup>13</sup>C-NMR (75 MHz, D<sub>2</sub>O) δ = 206.5 (s, C-4), 95.6 (d, C-1,  $^2J_{C-1,P} = 6.6$  Hz), 76.0 (s, C-3), 75.3 (d, C-2,  $^3J_{C-2,P} = 8.8$  Hz), 70.7 (s, C-5), 13.4 (s, C-6)

<sup>31</sup>P-NMR (121 MHz, D<sub>2</sub>O) δ = -11.85 (d, 1 P, P $\alpha$ ), -13.65 (d, 1 P, P $\beta$ ),  $^2J_{P\alpha,P\beta} = 19$  Hz

ESI-MS (negative mode): m/z = 532 [M(ketoform)-H]<sup>-</sup>; m/z = 550 [M(hydrate)-H]<sup>-</sup>

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FUKUI JUICHI

(74) Representative:

(54) NOVEL SYNTHESIS OF  
5-FLUOROURIDINE-5'-  
DIPHOSPHATE GLUCOSE

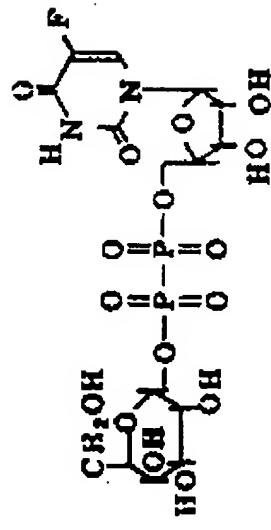
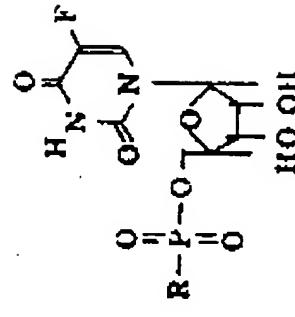
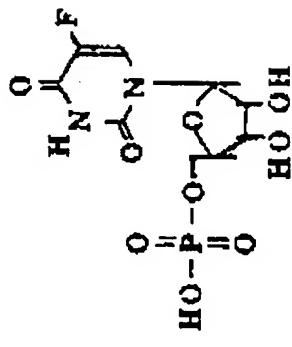
(57) Abstract:

PURPOSE: To synthesize the titled compound useful as a carcinostatic agent in high yield in large scale, by reacting 5-fluorouridine-5'-phosphate with glucose-1-phosphate in the presence of a cyclic secondary amine or an aromatic amine.

CONSTITUTION: 5-Fluorouridine-5'-phosphate amide shown by the

formula I is reacted with a cyclic secondary amine or an aromatic amine in the presence of a condensation agent such as carbonylimidazole, etc. in an alcohol such as t- butanol, etc. to give firstly 5-fluoro-uridine-5'-phosphate amide shown by the formula II, an intermediate. The intermediate is not isolated, and directly reacted with glucose-1-phosphate in a solvent preferably pyridine at 10W50°C for 1W5 days, to give the desired compound shown by the formula III.

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## Bibliographic Information

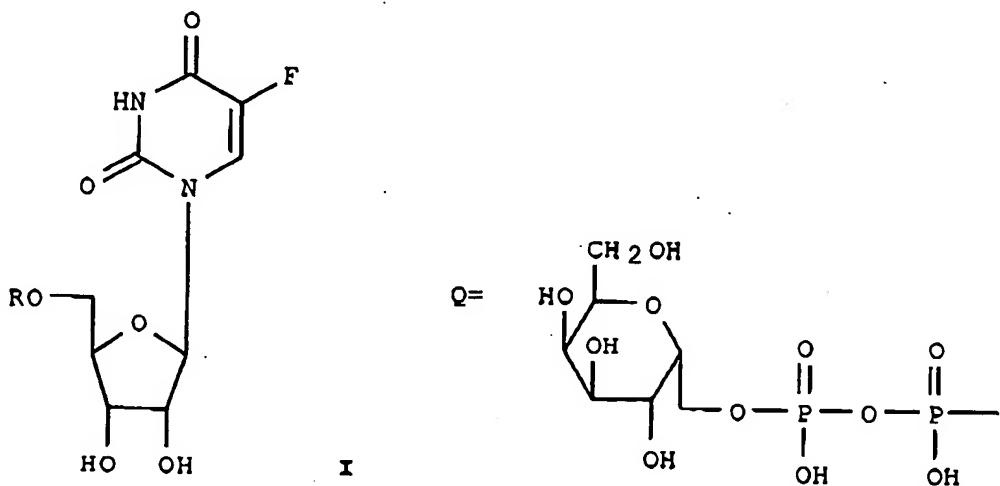
**Preparation of 5-fluorouridine 5'-phosphate derivatives as anticancer agents.** Tanaka, Toshio; Matsukawa, Akira. (Fuso Pharmaceutical Ind, Japan). Jpn. Kokai Tokkyo Koho (1995), 9 pp. CODEN: JKXXAF JP 07233187 A2 19950905 Heisei. Patent written in Japanese. Application: JP 94-161173 19940713. Priority: JP 93-350129 19931229. CAN 124:117893 AN 1996:73170 CAPLUS (Copyright 2004 ACS on SciFinder (R))

## Patent Family Information

<u>Patent No.</u>	<u>Kind</u>	<u>Date</u>	<u>Application No.</u>	<u>Date</u>
JP 07233187	A2	19950905	JP 1994-161173	19940713
<u>Priority Application</u>				
JP 1993-350129		19931229		

## Abstract

The title compds. [I; R = (HO)2P(O), Q] were prepd. by reaction of 5-fluorouridine with a phosphoric acid source in the presence of a microbial enzyme of *Serratia marcescens* to afford 5-fluorouridine 5'-phosphate I [R = (HO)2P(O)] and reaction of 5-fluorouridine 5'-phosphate with a galactose source and a phosphoric acid source in the presence of a microbial enzyme of *Candida saitoana* to afford I (R = Q). These compds. in vitro showed selective cytotoxic activity for cancer cells such as transformed 3T3, mouse tumor cells (B16-FO, LL/2, NS-1, X63-Ag8, SP2/0), human cancer cells (HeLa229, K-562, and MOLT-3), and in particular had a reduced toxicity for normal cells such as human skin fibroblast cell NHDF and mouse fibroblast cell 3T3. In particular, I (R = Q) had a reduced toxicity for normal cells such as human skin fibroblast cell NHDF and mouse fibroblast cell 3T3. Pharmaceutical formulations such as granule, capsule, and tablet contg. I (R = Q) were given.



## Patent Classifications

Main IPC: C07H019-10. Secondary IPC: A61K031-70; C12P019-30. Index IPC: C12P019-30; C12R001-425;

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C12P019-30; C12R001-72.

**Indexing – Section 33-9 (Carbohydrates)**

Section cross-reference(s): 1, 63

**Candida saitoana**

**Fermentation**

**Phosphorylation, biological**

**Serratia marcescens**

(prepn. of anticancer fluorouridine phosphate and diphosphate galactose by enzymic phosphorylation and/or galactosylation of fluorouridine with *Serratia marcescens* and/or *Candida saitoana*)

**Neoplasm inhibitors**

(prepn. of fluorouridine phosphate and diphosphate galactose as anticancer agents)

**Glycosidation**

(galactosidation, prepn. of anticancer fluorouridine phosphate and diphosphate galactose by enzymic phosphorylation and/or galactosylation of fluorouridine with *Serratia marcescens* and/or *Candida saitoana*)

**796-66-7P, 5-Fluorouridine 5'-phosphate**

**92748-40-8P**

**Role:** BAC (Biological activity or effector, except adverse); BPN (Biosynthetic preparation); BSU (Biological study, unclassified); RCT (Reactant); THU (Therapeutic use); BIOL (Biological study); PREP (Preparation); USES (Uses); RACT (Reactant or reagent)

(prepn. of anticancer fluorouridine phosphate and diphosphate galactose by enzymic phosphorylation and/or galactosylation of fluorouridine with *Serratia marcescens* and/or *Candida saitoana*)

**59-23-4, Galactose, reactions**

**316-46-1, 5-Fluorouridine**

**Role:** RCT (Reactant); RACT (Reactant or reagent)

(prepn. of anticancer fluorouridine phosphate and diphosphate galactose by enzymic phosphorylation and/or galactosylation of fluorouridine with *Serratia marcescens* and/or *Candida saitoana*)

## Supplementary Terms

***Serratia marcescens* enzymic phosphorylation; *Candida saitoana* enzymic phosphorylation galactosylation; fluorouridine phosphate prepn anticancer**